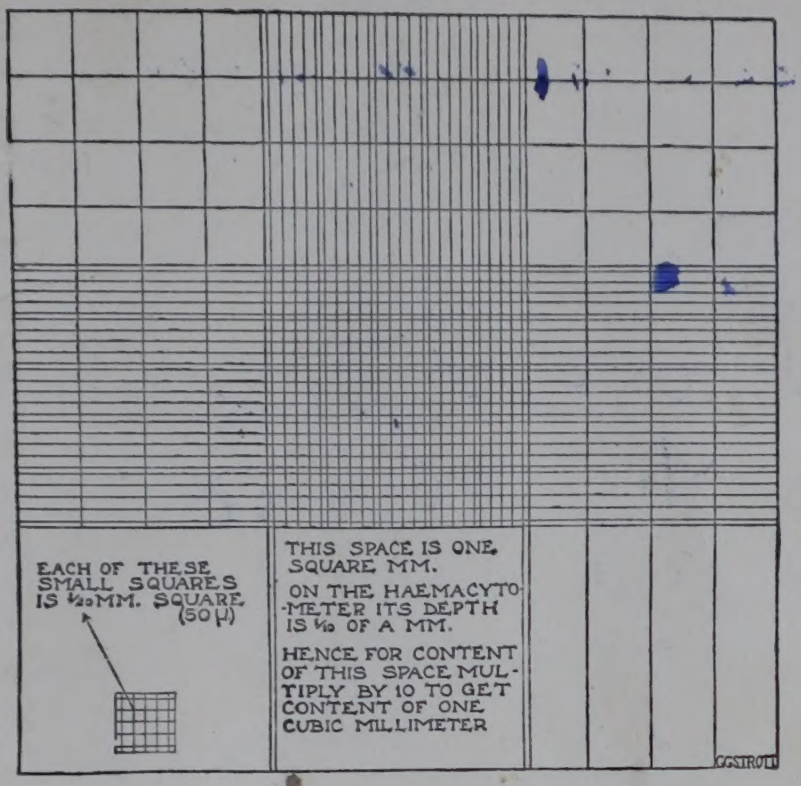
CFTRI-MYSORE
58
Practical bacter



The Improved Neubauer Ruling. Explanation of the Squares. The entire ruled area is a square, each side 3 mm. long. It is made up of 9 "large" squares, each 1 mm. square, which are subdivided for convenience in counting leukocytes.

The central square millimeter is subdivided into 400 "smallest" squares, each 0.050 mm. square. By making each fifth verticle and horizontal line a double line, these smallest squares have been segregated into 25 groups of 16 squares each.

The unit in estimating the leukocyte or red cell count of blood is the cubic millimeter, which is $\frac{1}{1000}$ of a cubic centimeter.

In making a leukocyte count, blood is drawn up in the white cell pipet usually to the 0.5 mark, and diluting fluid to the 11 mark above the bulb. This gives a dilution of 1 in 20.

With the $\frac{2}{3}$-inch objective, all the leukocytes in 4 (or 5) of the large (1-mm.) squares are counted and the average taken. Since the depth of the chamber is $\frac{1}{10}$ of a mm., this average figure is multiplied by 10 to get the number in 1 cu. mm. This product is then multiplied by 20 (the dilution) to get the number in 1 cu. mm. of blood.

Example: If an average of 90 leukocytes were counted in each 1 sq. mm., $90 \times 10 \times 20 = 18{,}000$, the number of leukocytes in 1 cu. mm. of blood.

To count the red cells, blood is drawn up in the red cell pipet to the 0.5 mark, and diluting fluid to the 101 mark. This gives a dilution of 1 in 200. With the $\frac{1}{6}$-inch objective the red cells are counted in 5 groups of 16 smallest squares each—80 in all. Since the central square millimeter contains 400 such squares, $\frac{80}{400}$ or $\frac{1}{5}$ of the sq. mm. has actually been counted. This number must be multiplied by 5 to get the number on 1 sq. mm. of the chamber. This product is multiplied by 10 to get the number in 1 cu. mm. and this again by 200, the dilution.

If 450 cells were counted: $450 \times 5 \times 10 \times 200$, or $450 \times 10{,}000 = 4{,}500{,}000$—the number in 1 cu. mm. of blood.

It is best to make a second preparation and record the average of the two counts.

PRACTICAL BACTERIOLOGY, HEMATOLOGY, AND PARASITOLOGY

58 (1) bacteria
(2) blood
(3) parasites
(4) protozoa
(5) helminths
(6) worms
(7) laboratory techniques
(8) medical entomology
(9) insects (10) poisonous

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Practical Bacteriology, Hematology, and Parasitology

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Tenth Edition

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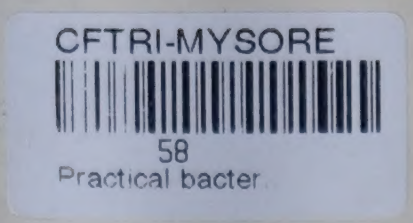
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Dedicated

to the Memory of

Dr. Mildred Clark Clough

Preface

Approximately 40 years ago the manuscript of the first edition of this manual was being written to cover the various fields of clinical microscopy in a single book. At that time it was the practice to have bacteriology, helminthology, protozoology, hematology, mycology, and urinalysis treated separately, so that there was inadequate correlation between the laboratory examinations in these fields and clinical diagnosis.

In each new revision more space has been allotted to the interpretation and diagnostic significance of the various laboratory procedures, and in this edition we have tried to correlate still further the data obtained by these examinations with the clinical picture. We believe that this is facilitated by bringing together in one book consideration of all the available types of laboratory examinations. Assistance in the selection of appropriate tests may be easily obtained by consulting Laboratory Procedures Useful in Diagnosis, Indexed by Diseases.

Following the Spanish-American War, interest in the study of tropical diseases was aroused, and books on the laboratory diagnosis of such diseases were being written. These, however, rarely coördinated the common diseases of temperate climates with those designated "tropical." In fact, the term "exotic diseases"—"Pathologie Exotique" of LeDantec—has seemed more appropriate than "Tropical Diseases" of Manson, for those diseases rarely or never seen in the clinics of Paris, London, or New York. Along with the first edition, successive editions followed the plan of giving equal consideration to the etiology of exotic diseases and those common to temperate climates.

Fortunate for the first edition was the assistance of Dr. Charles Wardell Stiles, later head of medical zoology in the Naval Medical School. Both here and at Johns Hopkins he covered the entire field from helminthology, his specialty, through protozoology, entomology, and herpetology, and he not only taught human parasitology but also illustrated this subject with comparative parasitology. Dr. Stiles was an international authority on zoologic nomenclature, and every zoologic name used in the first edition had his approval. Successive editions had the advice and coöperation of the officers of the Hygienic Laboratory (National Institute of Health) as well as of those attached to the Naval Medical School. In the present tenth edition we have revisions coming from these two institutions.

In the study of the filtrable viruses the clinical manifestations of the disease in man and experimental animals play a more important part in diagnosis than is the case in other conditions. In hematology a concise presentation of the symptomatology is needed as well as details of chemical and microscopic technic. The

symptomatology of certain diseases has been stressed if the material is to be found only in the recent literature or if, from the rare or exotic nature of the disease, it is dealt with inadequately or not at all in the standard textbooks.

We have continued to bear in mind the needs of the man in tropical or remote fields, who does not have access to well-equipped libraries or laboratories.

The great impetus given to epidemiology by World War II has resulted in a recognition of the necessity of having specialists in many fields, and the authors of this edition have been fortunate in obtaining recognized specialists to revise the subjects in their fields.

Revision of the section on Bacteriology has been done by Dr. Sara E. Branham of the National Institute of Health. This section has been almost completely rewritten. The Classification of Bacteria is based on that which will appear in the forthcoming Sixth Edition of Bergey's Manual. A number of new procedures have been described, such as determining Lancefield's groups of streptococci, newer methods of typing pneumococci and meningococci, Frobisher's chick method of determining the virulence of diphtheria bacilli, new methods of studying the enteric bacteria, and the use of the developing hen's egg for cultivation of bacteria, rickettsiae, and viruses. Sections on the Gram-negative bacteria and on the Gram-positive spore-bearing anaerobes have been extensively revised, and those dealing with the acid-fast bacteria and the lactobacilli completely rewritten.

The organisms causing actinomycosis and other related infections, formerly included under Mycology, have been discussed in Chapter 4 in connection with the acid-fast bacteria, as has also *Erysipelothrix rhusiopathiae*. The lactobacilli have been placed in Chapter 4 also, with other Gram-positive bacilli, since they are no longer considered primarily of the gastrointestinal tract. Glanders, on the other hand, has been transferred from Chapter 4 to 5 among the Gram-negative rods. The vibrio of cholera and the genus *Bacteroides* have also been placed in Chapter 5. Several new groups of bacteria have been added: Listeria, *Streptobacillus moniliformis* and pleuropneumonia-like organisms, and *Actinobacillus lignieresi*.

For advice concerning classification and nomenclature we are indebted to the Editorial Board in charge of the Sixth Edition of Bergey's Manual: Professor E. G. D. Murray, Lt. Colonel A. Parker Hitchens, and Professor Robert S. Breed, Chairman.

Acknowledgment is here made of the aid given by Dr. John H. Hanks in supplying material for the revision of the discussion of the acid-fast bacteria.

Obligation is here expressed to the many members of the staff of the National Institute of Health, who were unstintingly generous with advice and information for the section on Bacteriology. Among these special thanks should be given to Dr. Francis A. Arnold, Jr., Dr. James J. Griffitts, Dr. Albert V. Hardy, Dr. Carl L. Larson, Dr. Mark P. Schultz and Dr. Elizabeth Verder for invaluable help in their respective fields. Special thanks are due Miss Laura C. McCarty of this Institute for aid in preparation of the manuscript.

In Chapter 6, Spirochetes, the treponemiases have been discussed at considerable length. The discovery in 1938 that pinta, an important disease of the Indians of

the New World, is caused by a treponeme rather than a fungus, has broadened the field of these spirochetal diseases. There are still many unsolved problems as to the relationship of yaws and syphilis, among which is the explanation of the disappearance of yaws in our Southern States where it was introduced through thousands of cases among the African slaves.

In connection with spirochetal diseases, we have had the advice of Rear Admiral H. W. Smith (MC) USN, who was the editor of the seventh edition.

The section on rickettsial diseases, written by Dr. Norman H. Topping, of the U.S. Public Health Service, includes descriptions of Q fever and tsutsugamushi disease. He has also described the technic of cultivation of rickettsiae in eggs, the preparation of antigens from infected yolk sacs, and the soluble antigens released from certain rickettsiae by diethyl ether. For the photomicrographs of rickettsiae taken with the electron microscope we are indebted to Dr. R. W. G. Wyckoff. We also thank Dr. R. E. Dyer, Director of the National Institute of Health, for criticism of the manuscript.

The chapter on Medical Mycology has been revised by Lt. Comdr. Robert J. Goodlow H(S) USNR, Naval Medical School, NNMC. New material added includes recent advances regarding coccidioidomycosis and histoplasmosis, and a summary of the present status of antibiotics. New original illustrations have been added. We are indebted to Dr. C. W. Emmons, of the National Institute of Health, for advice and criticism of this section.

The section on Serologic Tests for Syphilis has been revised by Lt. Genevieve Stout H(W) USNR, Naval Medical School, NNMC. We are indebted to Dr. Reuben Kahn for a review of the section dealing with the Kahn flocculation test. The technic of the Wassermann reaction of the former edition has been replaced by the Kolmer complement-fixation test. We thank Dr. John Kolmer for a review of this section. A section has been added regarding the use of the cardiolipin antigen. For special information regarding this and for a review of this section we are indebted to Dr. Mary Pangborn, Dr. J. J. Mahoney, and Mr. A. Harris.

The chapter on Filtrable Viruses has been rewritten by Dr. Paul W. Clough. New material has been added, particularly regarding poliomyelitis, the encephalitides, lymphogranuloma inguinale, and influenza, and sections on infectious hepatitis and primary atypical pneumonia.

The short chapter on Bacteriology of Water and Milk contained in the former edition has been omitted to make room for more important material, but the essential data, particularly regarding the coliform organisms, have been included by Dr. Branham in preceding chapters.

The chapters on Preparation of Media and Reagents and Staining Methods and Special Procedures were prepared by Lt. Comdr. Paul V. Wooley (MC) USNR, Naval Medical School, NNMC, with the advice and help of Dr. Branham. These have been transferred to Part I, although for convenience some special procedures have been incorporated elsewhere. This has been radically revised, obsolete material removed, and only the most useful modern methods retained.

Revisions have been made in Part II dealing with Hematology, particularly in

pernicious anemia, sprue, and the group of hemolytic anemias. Among additional procedures discussed are: sternal marrow biopsies and punctates; new work on human blood groups and methods for their determination; the Rh factor and its relation to transfusion reactions and erythroblastosis fetalis; "cold" autohemagglutinins and their occurrence in primary atypical pneumonia; vitamin K deficiency, tests for prothrombin time, and its relation to bleeding in obstructive jaundice and hemorrhage of the newborn; preservation of blood for transfusions, and substitutes for blood.

Part III, Parasitology, has been rewritten: Medical Protozoology by Comdr. Elmer M. Bingham (MC) USNR; Medical Helminthology by Comdr. Trenton K. Ruebush H(S) USNR; and Medical Entomology by Lt. Comdr. William J. Perry H(S) USNR, all of the Naval Medical School, NNMC. Much additional material has been added, particularly important being new work on malaria, schistosomiasis, filariasis, DDT, and arthropod transmission of disease. A major addition is the large number of illustrations, practically all from new and original drawings or photographs. These include diagrammatic drawings illustrating the life cycle of the more important parasites. Colored plates illustrating malarial parasites in thin and thick blood films will, we believe, add greatly to the value of this section.

We are indebted to Lt. Comdr. Nancy H. Wheeler H(W) USNR, Naval Medical School, for reading and making many constructive criticisms of this entire section; to Dr. F. C. Bishop, Bureau of Entomology and Plant Quarantine, U.S.D.A., for reading the section on Medical Entomology; to Lt. D. E. Howell H(S) USNR, Naval Medical School, for advice in preparing the section on Medical Entomology, particularly the part dealing with the arachnids; and to Lt. (jg) J. R. Borland H(S) USNR, Naval Medical School, for preparing the photomicrographs used in the chapter on Medical Parasitology.

In Part IV, Chapter 35, Examination of the Cerebrospinal Fluid and of Fluid from the Serous Cavities, has been extensively revised and obsolete material omitted. We have substituted the new improved method of preparing colloidal gold solution now in use at the Naval Medical School laboratories, and for this we thank Lt. Comdr. Oris Western H(S) USN.

In Chapter 36 improved methods have been substituted for the determination in the blood of nonprotein nitrogen, urea nitrogen, uric acid, inorganic phosphorus, alkaline phosphatase, and serum protein. Among new procedures are the use of the photoelectric colorimeter, and methods for determining acid phosphatase, cholesterol esters, sulfonamides, and thiocyanate. We are indebted to Dr. Mary V. Buell for advice and information concerning procedures used in the Chemical Laboratories of the Medical Clinic of the Johns Hopkins Hospital. We also thank Comdr. J. J. Englefried H(S) USNR, for refinements of the Bogen method of determining alcohol in the blood.

Tests of renal function have been revised. In Chapter 42 we have added the cephalin-cholesterol flocculation test and the hippuric acid synthesis test, and have omitted several less important or less frequently used procedures.

Chapter 44, Vitamins as Specific Food Factors: Their Nutritional Significance

and Deficiency Effects, has been entirely rewritten by Lt. Comdr. M. Pijoan (MC) USNR, Naval Research Institute, NNMC. In view of the importance and current interest in this subject, a brief bibliography has been included.

In the Appendix several sections have been omitted to make room for more pertinent material. Sections B, C, and E of the former edition have been transferred to other parts of the book. Present Section G, Important Diseases and Injuries Due to Toxic Plants, has been revised and extended. For this new material we are indebted to Col. Richard P. Strong (MC) USA, who kindly made available material from the seventh edition of "Stitt's Diagnosis, Prevention and Treatment of Tropical Diseases," of which the chapter on poisonous plants had been revised by Professor Elmer D. Merrill, Director of the Arnold Arboretum. Present Section H, Laboratory Procedures Useful in Diagnosis, Indexed by Diseases, has been revised and extended.

We are indebted to Comdr. Emil Bogen (MC) V(S), USNR, for many suggestions as to revisions and additions in all sections of the book.

We wish to thank Captain H. Lamont Pugh (MC) USN, Medical Officer in Command, Naval Medical School, NNMC, for his continued interest in this book and for his kindness in making available the many illustrations and the large amount of material borrowed from the Naval Medical School.

We are especially indebted to Capt. Otis Wildman (MC) USN (Ret.), Director of Laboratories, Naval Medical School, NNMC, who has supervised the work done by the various authors from the Naval Medical School. Without his constant interest, constructive criticism and active collaboration much of this revision would have been impossible.

We are greatly indebted to the Medical Illustration Department of the Naval Medical School, under the direction of Lt. Comdr. Leon Schlossberg H(S) USNR, for the many new illustrations in the chapters Medical Parasitology and Medical Mycology. Lt. Annette Conry H(W) USNR drew two of the colored plates used in the section on Bacteriology and the thick films of malaria parasites. She also drew the plates of intestinal protozoa. The color plate of the thin film of malaria parasites was prepared under the direction of Captain James J. Sapero (MC) USN, to whom we are greatly indebted. This plate first appeared in the sixth edition of Strong: "Stitt's Diagnosis, Prevention and Treatment of Tropical Diseases," from which it is borrowed. James Garthwaite, Pharmacist's Mate, third class, made the majority of the illustrations for the chapter on Medical Entomology.

The editor also acknowledges the invaluable assistance of Comdr. Trenton K. Ruebush in editing the book, particularly Parts I and III, and in coördinating the work of the authors in the National Institute of Health and the Naval Medical Center, who have contributed to it.

E. R. Stitt
Paul W. Clough
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PART I

Bacteriology

By Sara E. Branham, M.D., Ph.D.

Chapters 1–6
By Sara E. Branham, M.D., Ph.D.

Chapter 7
By Norman H. Topping, M.D.

Chapter 8
By Paul W. Clough, M.D.

Chapter 9
By Robert J. Goodlow, Ph.D., Lieutenant Commander II (S) USNR

Chapter 10
By Paul W. Clough, M.D.
and Genevieve Stout, Lieutenant H (W) NNMC

Chapters 11–12
By Paul V. Wooley, Lieutenant Commander (MC) USNR
and Sara E. Branham, M.D., Ph.D.

CHAPTER 1

Study and Identification of Bacteria

General Considerations

Classification. The classification of bacteria has always been a difficult and complex problem, and there is still a difference of opinion in regard to the relationships and nomenclature of many of them. Morphologic characters are of fundamental importance in all classifications, yet in the case of bacteria few structural details can be made out, and these alone do not suffice to differentiate certain organisms which are otherwise very different. It is necessary, therefore, to utilize other characteristics, such as their metabolic activities, antigenic properties, and pathogenicity for animals. Although these characteristics are exceedingly important, particularly the antigenic structure, they are sometimes variable in the members of a certain species or even in a single strain under different conditions. Classification of the viruses is particularly incomplete.

With the exception of the genus *Salmonella* the classification given on pp. 4–8 is taken from the Sixth Edition of Bergey's Manual of Determinative Bacteriology. This is the most comprehensive work on microbiologic taxonomy, and, although it is not official, its use is constantly becoming more extensive and its terminology more familiar. Only such orders, families, tribes, and genera are included in the following condensation as are of medical interest. The Roman numerals correspond to those of the complete classification in Bergey's Manual. Those species of each genus which are apt to be encountered in medical bacteriology are listed, together with their older and common names. With the genus *Salmonella* the specific names recommended by the Nomenclature Committee of the International Association of Microbiologists has been adopted. The rules governing zoologic nomenclature are discussed in Chapter 16 (Medical Parasitology). The main points in regard to which botanic nomenclature differs are: (1) In cases in which strict adherence to the principle of priority would result in substituting an unfamiliar generic name for one in general usage, it is provided that the latter name may be "conserved." (2) The names of orders end in "ales"; suborders, in "ineae"; families, in "aceae"; subfamilies, in "oideae"; tribes, in "eae"; and subtribes, in "inae." (3) Specific names which are taken from the name of a person may begin with a capital, although Bergey has followed the zoologic rule, beginning all specific names with a small letter. The scientific name of a microörganism should be given in italics, whereas the common name is not. A generic name may be abbreviated only when it has already appeared fully in the context. It is necessary to follow this rule to avoid confusion since there is no official list of abbreviations. The rickettsiae and viruses are not included in this condensed classification.

Classification of Bacteria of Medical Importance

Class: Schizomycetes

Order I. *Eubacteriales*
- Family II. Pseudomonadaceae
  - Tribe I. Pseudomonadeae
    - Genus I. *Pseudomonas*
      - *P. aeruginosa* (*P. pyocyanea*)
  - Tribe II. Spirilleae
    - Genus I. *Vibrio*
      - *V. comma* (*V. cholerae*)
      - *V. metchnikovi*
    - Genus VI. *Spirillum*
      - *S. minus*
- Family IV. Rhizobiaceae
  - Genus III. *Chromobacterium*
    - *C. violaceum* (*B. violaceus*)
    - *C. ianthinum*
- Family V. Micrococcaceae
  - Genus I. *Micrococcus*
    - *M. pyogenes* variety *aureus* (*Staphylococcus aureus*)
      - variety *albus* (*Staphylococcus albus*)
    - *M. citreus* (*Staphylococcus citreus*)
    - *M. epidermidis*
    - *M. aerogenes*
    - *M. anaerobius*
  - Genus II. *Gaffkya*
    - *G. tetragena* (*Micrococcus tetragenus*)
  - Genus III. *Sarcina*
    - *S. ventriculi*
    - *S. flava*
    - *S. lutea*
- Family VI. Neisseriaceae
  - Genus I. *Neisseria*
    - *N. gonorrheae* (Gonococcus)
    - *N. meningitidis* (Meningococcus)
    - *N. catarrhalis*
    - *N. sicca*
    - *N. perflava*
    - *N. flava*
    - *N. subflava*
    - *N. flavescens*
  - Genus II. *Veillonella*
    - *V. parvula*
    - *V. gazogenes*
- Family VII. Lactobacteriaceae
  - Tribe I. Streptococceae
    - Genus I. *Diplococcus*
      - *D. pneumoniae* (Pneumococcus)
    - Genus II. *Streptococcus*
      - *S. pyogenes* (*Streptococcus hemolyticus*)
      - *S. equi*

S. equisimilis
S. agalactiae (*S. mastitidis*)
S. salivarius
S. mitis (*Streptococcus viridans*)
S. bovis
S. equinus
S. faecalis (Enterococcus)

Tribe II. Lactobacilleae
Genus I. *Lactobacillus*
L. acidophilus
L. bifidus
L. bulgaricus
L. casei
L. brevis
L. arabinosus
L. fermenti

Family VIII. Corynebacteriaceae
Genus I. *Corynebacterium*
C. diphtheriae
C. pseudodiphtheriticum (*B. hoffmannii*)
C. xerose
Genus II. *Listeria*
L. monocytogenes
Genus III. *Erysipelothrix*
E. rhusiopathiae

Family IX. Achromobacteriaceae
Genus III. *Alkaligenes*
A. faecalis

Family X. Enterobacteriaceae
Tribe I. Escherichieae
Genus I. *Escherichia*
E. coli
E. freundii (*Citrobacter*)
Genus II. *Aerobacter*
A. aerogenes
A. cloacae
Genus III. *Klebsiella*
K. pneumoniae (Friedländer's bacillus)

Tribe III. Serrateae
Genus I. *Serratia*
S. marcescens
S. kiliense

Tribe IV. Proteae
Genus I. *Proteus*
P. vulgaris
P. morgani

Tribe V. Salmonelleae
Genus I. *Salmonella*
S. paratyphi A (*B. paratyphosus A*)
S. paratyphi B (*S. schottmuelleri*) (*B. paratyphosus B*)
S. paratyphi C (*S. hirschfeldii*) (*B. paratyphosus C*)
S. choleraesuis (*B. suipestifer*)

S. enteritidis (*B. enteritidis*)
S. typhimurium (*B. aertrycke*)
S. typhi (*Eberthella typhosa*) (*B. typhosus*)

Genus II. *Shigella*
S. dysenteriae (Shiga's bacillus)
S. ambigua (Schmitz's bacillus)
S. paradysenteriae (*B. dysenteriae*, Flexner) (14 types)
S. alkalescens
S. madampensis (*S. dispar*)
S. ceylonensis (*S. dispar*)
S. sonnei

Family XI. Parvobacteriaceae
Tribe I. Pasteurelleae
Genus I. *Pasteurella*
P. avicida (*P. aviseptica*)
P. muricida (*P. muriseptica*)
P. suilla (*P. suiseptica*)
P. cuniculicida (*P. lepiseptica*)
P. pestis (*B. pestis*, plague bacillus)
P. pseudotuberculosis
P. tularensis

Genus II. *Malleomyces*
M. mallei (*Actinobacillus mallei*, *B. mallei*)
M. pseudomallei (*B. whitmori*)

Genus III. *Actinobacillus*
A. lignieresi

Tribe II. Brucelleae
Genus I. *Brucella*
B. melitensis
B. abortus
B. suis
B. bronchiseptica

Tribe III. Bacteroideae
Genus I. *Bacteroides*
B. fragilis
B. melanogenicus
B. funduliformis
B. serpens

Genus II. *Fusobacterium*
F. plautivincenti

Tribe IV. Hemophileae
Genus I. *Hemophilus*
H. influenzae
H. suis
H. hemolyticus
H. parainfluenzae
H. pertussis
H. conjunctivitidis
H. ducreyi

Genus II. *Moraxella*
M. lacunatus (Morax-Axenfeld)

Genus III. *Noguchia*
N. granulosis

Genus IV. *Dialister*
D. pneumosintes
Family XIII. Bacillaceae
Genus I. *Bacillus*
B. anthracis
B. cereus
B. subtilis
Genus II. *Clostridium*
Cl. fallax
Cl. septicum (*Vibrion septique*)
Cl. chauvoeii
Cl. novyii (*B. oedematiens*)
Cl. botulinum
Cl. sporogenes
Cl. histolyticum
Cl. bifermentans (*B. sordelli*)
Cl. perfringens (*B. welchii*)
Cl. tetani
Cl. tertium
Order II. *Actinomycetales*
Family I. Mycobacteriaceae
Genus I. *Mycobacterium*
M. tuberculosis, var. *hominis*
M. tuberculosis, var. *bovis*
M. phlei
M. lacticola
M. avium
M. paratuberculosis
M. leprae
M. leprae-murium
Family II. Actinomycetaceae
Genus I. *Nocardia*
N. madurae
N. asteroides
Genus II. *Actinomyces*
A. bovis (*A. israeli*)
Order V. *Spirochaetales*
Family I. Spirochaetaceae
Genus I. *Spirochaeta* (Ehrenberg, 1838)
S. plicatilis
Genus II. *Saprospira* (Gross, 1911)
S. grandis
Genus III. *Cristispira* (Gross, 1910)
C. balbianii
Family II. Treponemataceae
Genus I. *Borrelia* (Swellengrabel, 1907)
B. gallinarum (*B. anserina*)
B. recurrentis
B. novyi
B. duttonii
B. berbera
B. carteri
B. vincentii

B. buccale
B. eurygyrata
B. bronchialis
B. theileri

Genus II. *Treponema* (Schaudinn, 1905)
T. pallidum

Genus III. *Leptospira* (Noguchi, 1917)
L. icterohaemorrhagiae
L. hebdomadis
L. morsus-muris

Since the validity of any scheme of classification of bacteria depends upon a reasonable degree of constancy in morphology and in other properties, attempts have been made to minimize variations by prescribing standard conditions as to culture media, time of incubation, etc., under which they are to be grown for purposes of description. Under the influence of Koch, who insisted on the fixity of type, those which did not conform were regarded by most observers as contaminants or involution forms or were simply ignored. The occurrence of pleomorphism, however, must be recognized. In some bacteria this is very marked, especially in relation to age of culture and to type of medium.

Dissociation or Variation. Marked variations may occur in some of those characteristics of a species which were formerly regarded as of fundamental significance. The variations which at present seem to be of the greatest practical significance are those commonly included in the term "dissociation." For example, if a culture of the typhoid bacillus is carried through successive generations by streaking a single colony over an agar plate, colonies of two distinct types may be found: (1) *"Smooth" colonies* (S form), glistening, moist, rather viscid and homogeneous, with even round borders; and (2) *"rough" colonies* (R forms), with a dry, crinkled surface, a tough, granular consistency, and irregular margins. There may be colonies intermediate or mixed in type. Subcultures from typical colonies of each type usually reproduce the corresponding type. It is relatively easy to induce dissociation from a smooth to a rough type of colony. This may occur spontaneously in old stock cultures or may be induced by unfavorable conditions for growth, especially by adding immune serum to the medium. Bacteriophage is often potent in initiating the change. It is more difficult to secure a reversion from the rough to the smooth type, though this is possible in some cases. For example, a fixed type pneumococcus which has been dissociated will revert to a smooth fixed type if inoculated into



Smooth and rough colonies of the typhoid bacillus (*Salmonella typhi*); × 3. Culture on nutrient agar inoculated from an old broth culture. (Courtesy, Jordan and Burrows: Bacteriology, Philadelphia, W. B. Saunders Co.)

a mouse together with the specific carbohydrate haptene of that type. (See Pneumococcus.)

These alterations in the appearance of the colonies are accompanied by significant changes in the other characteristics of the organisms. The *smooth type* forms homogeneous suspensions with little tendency to spontaneous clumping, and shows coarse flocculation when specifically agglutinated. Motility or capsule formation, if characteristic of the species, is well marked. Such organisms are relatively virulent and resistant to phagocytosis, and are more effective for immunization because they contain all the antigens of the species.

The *rough type* forms granular suspensions. The organisms tend to clump spontaneously and form fine clumps when specifically agglutinated. They are less virulent and are susceptible to phagocytosis. They often lose their flagella or their capsules, and also the corresponding antigens, retaining only the somatic antigen. There may be changes in the character of the latter also. (See sections on the pneumococcus, proteus, typhoid and paratyphoid bacilli.) They are relatively ineffective for immunization. The individual cells may show changes in morphology. They may be larger or smaller than the typical smooth strains, and may grow out into filamentous or branching forms.

Two other types of colonies are sometimes found. The "mucoid" type is large and moist. The "G" colonies of Hadley are very minute, relatively inert, and hard to propagate. They are often composed of tiny granular filtrable forms.

Dissociation occurs in many groups of bacteria, but the dissociants do not always have the exact characteristics described above. Each group of bacteria is a law unto itself. Since this change from "smooth" (S) to "rough" (R) is usually accompanied by a loss of specific antigen, many workers prefer to speak of "specific" and "nonspecific" phases, since typical "roughness" or "smoothness" of colonies is not always apparent.

These changes have been demonstrated in the body as well as in the test tube. They introduce practical difficulties into the interpretation of agglutination tests and necessitate the careful selection of strains for the production of vaccines and serums.

Morphology. Little is known as to the finer structure of the bacterial cell. The development of the electron microscope is adding to our knowledge, but much remains to be learned. Definite cell walls have been shown on certain bacteria by some workers. On the whole it may be said that the bacterial cell is composed of an inner cytoplasmic mass, the endoplasm, and of an outer ectoplasm which makes up the cell membrane, the flagella, and the capsular material. It has been shown that in many species the chemical constitution and the antigenic properties of the ectoplasm and endoplasm are radically different. Usually the ectoplasmic structures are relatively well developed in virulent, actively growing cultures.

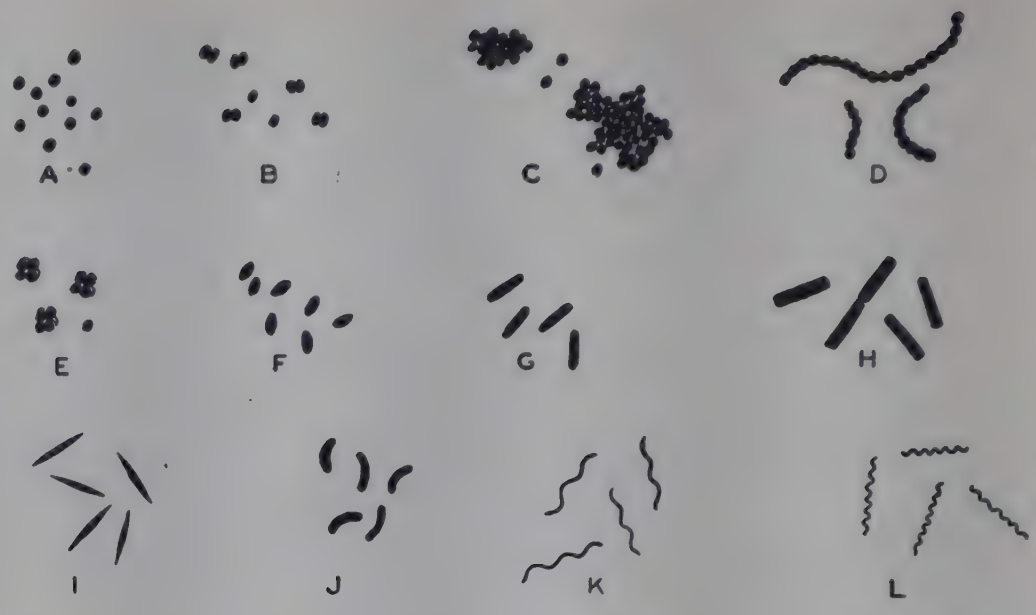
The presence of *nuclear material* in the endoplasm has been proved. The electron microscope has shown this in granules in some bacteria and concentrated in one mass in others. The structure of the "nucleus" evidently varies in different groups of bacteria.

Endospores are formed by certain species by a process of condensation and dehydration of the cell cytoplasm. They are round or oval, highly refractile structures which stain with difficulty and resist decolorization when stained. Their resistance to heat, desiccation, and unfavorable outside environment is obviously advantageous for the continued existence of the species. It also facilitates the isolation of such organisms in pure culture, since on heating to 80° C. for 30 minutes the spores survive whereas other forms are killed. Spores are formed, however, only in young, actively growing cultures under suitable environmental conditions.

The only spore-formers of medical interest are the anthrax bacillus and a few anaerobes: the bacillus of tetanus, of botulism, and the group causing gas gangrene.

Reproduction. The usual method of reproduction of bacteria is by binary transverse fission. Exceptionally, other methods have been observed.

Food Requirements. Bacteria contain no chlorophyll. Except for some of the sulfur bacteria, energy for growth must be obtained by oxidation or other chemical degrada-



Morphologic types of bacteria. (A) Micrococcus. (B) Diplococcus. (C) Staphylococcus. (D) Streptococcus. (E) Sarcina. (F) Coccobacilli. (G and H) Commonly occurring rod forms. (I) Fusiform bacilli. (J) Curved rods or vibrios. (K and L) Spiral forms. (Courtesy, Jordan and Burrows: Bacteriology, Philadelphia, W. B. Saunders Co.)

tion of materials in the fluid medium containing them. Their requirements differ extremely. For some species these are satisfied by the meager contaminations present in distilled water, while certain pathogens grow only under highly artificial conditions in media containing blood or tissue fluids. The filtrable viruses and the rickettsiae are still more exacting in that they will grow only in association with living cells. All species of medical interest require organic material of some sort in the medium as a source of nitrogen as well as energy.

In addition to the carbon, nitrogen (as amino acids or peptone), and mineral salts which enter into the chemical composition of the bacterial protoplasm, some species also require certain accessory growth factors which probably function as vitamins. The discovery by Lloyd that these substances may be adsorbed by cotton or paper through which media are filtered was of great practical importance in improving the quality of culture media. Little is known as to the nature or mode of action of such substances. They may be obtained from a variety of sources such as fresh blood, tissues, beef infusion, or potato juice.

Oxygen Requirements. Most bacteria depend for their source of energy on the oxidation of organic materials. Aerobes utilize the free oxygen in the medium. Obligate anaerobes must derive their oxygen from other sources: from reducible substances in the medium or from the products of cleavage of carbohydrates or proteins. It has been suggested that the inhibitory effect of free oxygen on obligate anaerobes is due to the toxic effect of peroxides which these organisms are unable to split. Such organisms may be made to grow in the presence of some free oxygen by adding to the medium a little sterile tissue or unheated potato both of which contain a catalytic ferment. Most aerobes are facultative anaerobes, but some, such as the plague bacillus and the tubercle bacillus, require abundant free oxygen. Some organisms, particularly on isolation, require a reduced oxygen tension and will not grow under either aerobic or strict anaerobic conditions. This is most easily secured by using a medium containing a reducing substance.

Metabolic Products. In the course of their active growth bacteria produce a great variety of substances. Some of these are enzymes which play an essential part in the nutrition of the organism. Proteolytic enzymes are formed by a great variety of bacteria. These may be demonstrated by testing the capacity of a culture to liquefy gelatin, coagulated blood serum, or egg white, and are important in classification. The capacity to split carbo-

hydrates and to ferment various sugars and alcohols also depends on special enzymes. As a rule these activities are fairly constant and specific, and are important in identification, particularly of members of the enteric group. The activities of some of these bacterial enzymes are of great industrial importance, such as the alcoholic fermentation of sugar, the oxidation of alcohol to acetic acid, and the cleavage of corn starch with the production of butyl alcohol and acetone.

As a result of these metabolic activities, various by-products accumulate in the culture media, such as acids, alcohols, peroxides, amino acids, and other protein decomposition products, which inhibit the growth and eventually bring about the death of the organisms which produce them. The by-products of one species usually form the pabulum for another, until under natural conditions the dead bodies and refuse of plant and animal origin are completely broken down into simple substances (carbon dioxide, nitrates, water) which can be utilized by chlorophyll-containing plants and resynthesized into materials which can serve as food for animals. Bacteria thus help to maintain the "circulation" of carbon and nitrogen in usable form between the plant and animal kingdoms.

Toxic Substances. Pathogenic organisms also form various substances which are injurious to their host and which doubtless protect them more or less effectively from the antibacterial action of the defensive forces of the host. Among these are the specific soluble toxins, secreted by certain bacteria and liberated into the surrounding medium. Another type is met with in the specific soluble substances contained in the capsular material of pneumococci and Friedländer bacilli (*Klebsiella pneumoniae*). This appears to endow the organism with the capacity to resist phagocytosis and invade the tissues. Failure to form capsules is associated with loss of virulence. Bacteria form other "aggressive" substances which appear to be an integral part of their protoplasm and are liberated only on disintegration of the bacterial cell (the endotoxins). These are complex mixtures and may contain two or more distinct fractions, each possessing antigenic powers and each capable of stimulating the production of antibodies and of allergic reactions in the host, specific for itself. These antigens may be restricted in distribution to the one species of bacterium producing them, or one or more of them may be found also in related species or even in unrelated organisms, as in the case of the rickettsiae and *Proteus vulgaris*. The antigenic content of a strain may be altered if the culture becomes dissociated. These complex relationships have been studied especially in the enteric group and will be discussed in that section.

Factors Determining Infection. In general the occurrence of infection depends upon the balance between the invasive powers of the organism and the defensive forces of the host. The various means by which the host resists infection are discussed in a later chapter. If infection is to be established the invading organisms must possess an adequate degree of virulence. It is necessary that the number of invading organisms be adequate. Although in exceptional instances it is possible for a single organism to infect, as in the case of highly virulent anthrax bacilli or pneumococci in the rabbit, as a rule an appreciable number are required, and if the dosage is insufficient the invading organisms will be destroyed. A massive dose may infect even though the virulence is relatively low or the resistance of the host high. The invaders must also reach a suitable portal of entry into the body. Thus infection may be brought about readily if the typhoid bacillus reaches the intestine; whereas infection with a virulent hemolytic streptococcus would be more likely to occur through a skin abrasion than through the gastrointestinal tract.

A recognized fact in epidemiology is that in the spread of an epidemic certain individuals seem to escape infection, notwithstanding intimate exposure; some contract the disease in a mild form, while others suffer severely or die in the course of the epidemic.

As stated by Theobald Smith, the coöperation of two parasites (symbiosis) is to be thought of in the spread of epidemics, and this field is an important one. The best known example of the symbiosis of bacteria is the association of spirilla and fusiform

bacilli in Vincent's infection, and probably of anaerobic streptococci also in severe cases. Recognition of the association of the hog-cholera bacillus and the filtrable virus was an outcome of Dorset's work. The study of the connection between the influenza of swine and that of man is now being actively pursued. In both there is the frequent association of an organism of the Pfeiffer type. Shope, in his work with swine influenza, has shown that a bacillus of this type as well as a virus is required.

Adaptation. The severity of the symptoms that accompany an infection depends in some cases upon the capacity of the organism to injure the host on the one hand, and the effectiveness and promptness of the defensive forces of the host in limiting multiplication of the organisms and neutralizing their toxic products on the other. In other cases, in spite of the successful invasion of the host and continued growth of the organisms, the host suffers little or not at all. It is obviously advantageous to the parasite as well as to the host to live and multiply in the tissues of the host without seriously injuring the latter. An example of such an adaptation is found in infection of the rat with *Trypanosoma lewisi* which appears to be quite harmless to these animals. This state is approached in the case of the virus of simple herpes in man.

The bacteria constituting the "normal flora" of the upper respiratory passages and the intestinal tract behave as harmless commensals as long as they do not penetrate the mucous membranes. Some of them, however, may become pathogenic if they succeed in gaining a foothold in the tissues. The adaptation of bacteria to the host rarely reaches such a point that the bacteria can grow in the tissues without causing injury. A few individuals of more virulent species may persist for long periods in the tissues in a latent but viable state, particularly in the lymph nodes and spleen or in inflammatory scar tissue. Such dormant infections, however, constitute a constant danger to the host.

Methods of Study and Isolation

Collection of Specimens. Collected samples should represent the actual substance of the lesion, as far as possible uncontaminated by extraneous material. Containers should be suitable, clean, and sterile. Scanty material may be taken on sterile cotton swabs. Fluids should be collected in sterile tubes or bottles. Aseptic collection may be facilitated by the use of syringes. Material shipped requires careful packing to prevent loss from breakage or leakage and must conform with postal regulations. Specimens which dry rapidly or are susceptible to change should be delivered to the laboratory at once. Proper temperature should be maintained and examination performed as soon as possible after collection of the specimen. Directions for collecting and handling materials from various sources are given in detail in Chapters 30 to 41.

Microscopic Examination. Material in suspension may be examined in a wet-mount or hanging-drop preparation (see p. 362) by transmitted light or in a dark field (p. 914). For examination of fixed bacteria, the material is spread in a thin film on a slide or coverslip, dried in air, fixed (p. 362), stained (p. 362), and examined with an oil-immersion objective. Cells and other structures should be noted as well as bacteria. Laboratory diagnosis of some diseases whose causative organisms cannot be cultured readily rests almost wholly upon direct smear examination.

Isolation in pure culture is indispensable for the study of most bacteria.

Streaked Plates. This is a favorite method of obtaining isolated surface colonies. After having examined a stained film of the material to be cultured to determine approximately the number of organisms present and the degree of dilution necessary to obtain isolated colonies, a loopful of the material, diluted if necessary, is placed on the surface of an agar plate near one margin. As a spreader one may use a small sterile cotton swab or, preferably, a fine glass rod or platinum wire, the end of which is bent at a right angle about 2 cm. from the tip. Dilution is accomplished most rapidly by using small spreaders. In streaking plates with a glass rod several plates may be required to obtain discrete isolated colonies, while with a platinum wire the same result may be obtained with only

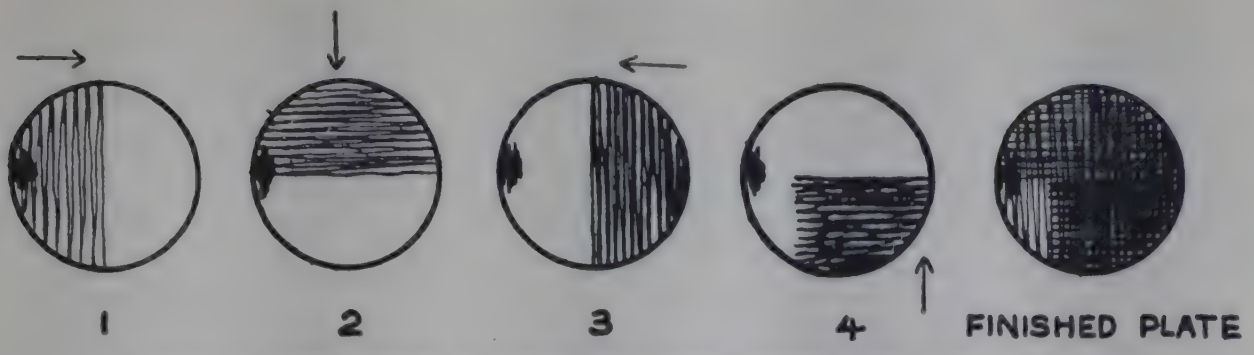


Diagram showing method of streaking a plate. Drawing, Naval Medical School.

two or three plates. The spreader is sterilized by heat and cooled, and with the bent tip the material is streaked over the surface of two or three agar plates in a series of parallel sweeps without sterilizing or re-inoculating the spreader. The weight of the handle of the spreader must be supported by the fingers so that the surface of the medium is not roughened. A method described by Rake (1934) for nasopharyngeal cultures usually requires only one plate. The tip of the swab is brought gently into contact with the surface of the plate and a smear is made on the agar over a very small area at one edge. Then a fresh sterile swab is taken, and with it the inoculum is spread over one-half of the plate, the excursions back and forth across the plate being made very close together and overlapping. A second fresh swab now spreads the material at a right angle for a fourth of the plate, and a final swab is used to finish covering the surface with a progressively decreasing inoculum spread at a right angle to the quarter previously streaked. A bent platinum loop or wire may be used in place of the swabs and sterilized after the inoculation of each area.

As a rule, after incubation, on one of the plates, if several have been prepared, the distribution will be such that discrete colonies are obtained, sufficiently separated from each other so that the characteristics of each type of colony can be recognized and individual colonies "fished" and subcultured for further study and identification. This can be done most conveniently by placing the plate on the stage of a dissecting microscope and touching the colony with the tip of a platinum needle while looking through the lens. As the needle is about to touch the colony, its point comes clearly into focus. It is well to support the right hand with the left in order to steady the needle. If the colony is large enough a second particle may be fished from it and rubbed up in a small loopful of water on a coverslip, examined for motility, spread, and then stained by Gram's method. Each colony examined should be clearly marked and numbered on the underside of the plate with a wax pencil and the same number affixed to each slide or culture tube subsequently used in studying this organism. If no dissecting microscope is available a hand lens may be used to examine the colony before it is marked and fished. It is important to wait for at least 48 hours of incubation (in the case of some bacteria at least seven days) for the development of colonies before discarding the culture.

For routine examination of most types of material from patients, blood agar plates should be used, or suitable special agar media, rather than plain agar. For feces we use SS agar (Salmonella-Shigella), bismuth sulfite, or MacConkey's medium. Urine, if heavily infected, should be diluted. If not, one drop should be spread on one half of the plate and five drops on the other half.

Poured Plates. These are used chiefly for making counts of viable bacteria. In such cases, serial dilutions of the material being studied are made quantitatively, and known amounts mixed with tubes of melted agar which are poured into the Petri dishes, allowed to harden, and incubated.

In using melted agar for plating it must be remembered that agar solidifies at a temperature slightly below 40° C. and does not melt until the temperature is raised nearly to the boiling point. Since the organisms to be cultivated may be injured by tempera-

tures over 44° C., it is necessary to work carefully and quickly to avoid premature solidification of the agar.

Agar Slants. Instead of using plates, material on a platinum loop may be stroked successively over the surface of four or five agar or blood agar slants, or successive dilutions may be made in the water of condensation of several blood agar tubes. The water of condensation is then made to flow once over the surface of the slants, and the tubes are incubated. Discrete colonies can be obtained which can be examined with a lens and fished without difficulty, although not so conveniently as from plates. Such a procedure may be useful in the field where transportation and handling of Petri dishes are not practicable.

Deep Agar Tubes. Anaerobic bacteria will not grow on the surface of plates and slants unless these are incubated in an atmosphere from which free oxygen has been removed. Such microörganisms are frequently isolated by mixing the inoculum in a deep tube of melted agar which is allowed to solidify. The colonies develop deep in the medium. Not all colonies found in deep agar are anaerobes. It must be remembered that many facultative anaerobes will grow under the same conditions.

There are special methods of cultivating anaerobes which will be discussed in Chapter 3.

Stock Cultures. The ideal method of preserving stock cultures is to dry them *in vacuo* from a frozen state. Freezing should be quick, drying complete, and the containing vials should be sealed *in vacuo* and stored at usual icebox temperature; e.g., the "lyophile process" of Flosdorf and Mudd. This convenient method preserves the biologic and immunologic properties which are often changed during the repeated subculturing of ordinary laboratory maintenance. The cultures are easily shipped in this dried state and may remain viable for many years.

If freezing and drying *in vacuo* is impracticable, ordinary cultures may be kept at below freezing temperatures and will remain viable for many months. The choice of medium depends on the microörganism, but, as a rule, a semisolid medium such as that described by Hitchens (1921) maintains most bacteria well.

Barber's Technic for Isolating a Single Bacterial Cell. This method is very valuable in securing indisputably pure cultures of bacteria, especially anaerobic spore-bearers. It is especially important as a preliminary step in the study of dissociation or possible mutational changes. Much practice is required to master the procedure, and the reader is referred to Barber's article for detailed technic (*Philippine Journal of Science,* 1914).

Methods of Identification

The most useful points to be noted for the purpose of identifying a culture after isolation are the following:

Morphology. Fundamental points are the shape, size, and arrangement of the organisms; the formation of pairs, packets, tetrads, chains, or palisade-like clusters, and the occurrence of polar bodies or metachromatic granules. In some cases (as plague bacilli on salt agar) characteristic involution forms occur. With many bacteria the age of the culture is an important factor in morphologic appearance.

Motility. A young (4- to 18-hour-old) culture should be used. A small drop is placed in the center of a coverslip, and this is mounted over the concavity of a hollow-ground slide. If this is not available, a ring of petroleum jelly is made on an ordinary slide, the drop placed in the middle of the ring, and a coverslip lowered onto it. This prevents evaporation and the production of fluid currents. Four- to 18-hour growth on an agar slant may be touched with the tip of a platinum needle and rubbed up in a drop of water or salt solution which is then mounted in a similar way. Examination is made with the light much cut down with the diaphragm. First the edge of the drop is brought into focus with the ⅔-in. (16-mm.) objective and then examination is made with the ⅙-in. (4-mm.) objective (not with the oil-immersion lens).

If truly motile, some of the bacteria will show progressive motility in various and diverse directions, and will move away from the place where they were first observed. This must be differentiated from motion due to currents in the fluid, which is characterized by the swarming of all the bacteria in the same direction, at varying speed; and from Brownian movement, a rapid oscillating motion of small amplitude, which may be observed with any minute inert particles in a liquid. Only a few of the organisms in the field may show true motility. Occasionally a strain may show little or none immediately after isolation but show active motility after several subcultures in broth have been made. Motility may be lost if a culture becomes dissociated.

Flagella. The number and the arrangement of the flagella in the case of motile organisms is also sometimes distinctive. An organism may have a single flagellum at one pole (monotrichous), flagella at each pole (amphitrichous), or many flagella all around them (peritrichous). The cholera vibrio, a monotrichous organism, moves at a rate of 0.030 mm. per second, while the typhoid bacillus which is peritrichous has a rate of movement about half as great—0.018 mm. The technical difficulties in staining flagella are so great that the procedure is seldom used for routine diagnosis.

Spore Formation. This has been discussed (p. 9).

Gram's Stain. Gram's stain is of fundamental importance in differentiation. Cultures not over 24 hours old, and preferably younger, must be used and the staining solutions must be efficient. A strain may lose its capacity to retain the stain after long-continued cultivation on artificial media. The method of staining by Gram's technic is described on p. 363.

Practically all pathogenic cocci are Gram-positive except the Neisseria. Practically all pathogenic bacilli are Gram-negative except the spore-bearers, the acid-fast bacilli, and the diphtheria group.

Special staining reactions (described in the special sections) are important in the recognition of certain organisms; notably capsule stains (pneumococcus, Friedländer bacillus), acid-fast stains (tubercle bacillus), and stains for polar bodies (diphtheria bacillus).

Cultural Characteristics

Single Colonies. The characteristics of individual colonies on agar or blood agar plates are important. The points to be noted are the size and shape, the degree of opacity, the character of the margin and of the surface (smooth or rough), the texture and consistency (moist and viscid or dry and tenacious), and the presence or absence of pigment. On blood agar the presence of hemolysis or of a greenish discoloration of the blood around the colony is very important in the differentiation of streptococci.

Broth cultures are useful in identification chiefly for tests for motility. Search should be made for turbidity, sediment, and pellicle or stalactite formation.

Potato cultures are useful only in special cases, particularly in stimulating pigment production. Pigment often develops well on Loeffler's serum medium or on starch agar.

Litmus milk cultures have been extensively used, but the information they yield may be obtained by other methods, particularly fermentation tests. Note successive changes in reaction, coagulation, liquefaction, and gas formation.

Gelatin. Liquefaction of gelatin is an important means of differentiation of bacteria in general, but is relatively unimportant in the study of pathogenic organisms except the cholera and proteus bacilli. If plates are used a room-temperature incubator (20° C. to 22° C.) is required. If one is available, deep stab cultures may be made, and the shape of the liquefied area noted as well as the presence or absence of liquefaction. If a low-temperature incubator is not available, or if the pathogens do not grow at low temperatures, the tube should be incubated at 37° C., and then immersed in ice water. Failure of the gelatin to solidify indicates that the organism is a liquefier. Liquefaction is effected by an extracellular ferment, gelatinase, which is present in bacteria-free filtrates of liquefying organisms.

Fermentation of Carbohydrates. Fermentation of carbohydrates is of great importance in the identification of many organisms. For ordinary routine work a few more common ones are chiefly used, but for special studies many other sugars, alcohols, and other carbohydrates are also employed. The occurrence of fermentation is indicated by the production of acid and in some cases of gas also. Culture media for studying fermentation should consist of a sugar-free base with the desired carbohydrate added, and usually an indicator. For many bacteria a simple peptone solution makes a satisfactory base either as a broth or as the semisolid medium of Enlows (1923). Other bacteria require a more complex pabulum, in which case meat infusion broth may be rendered sugar-free by fermentation with *Escherichia coli* (*B. coli*) and subsequent filtration. Small vials inverted in the tubes of broth, or special fermentation tubes, may be used if gas production is to be studied, though gas may be observed in semisolid agar as well as in stab cultures in solid media.

Indol. The production of indol (a decomposition product of tryptophane, one of the amino acids of protein) is characteristic of bacteria which are active in causing the decomposition of protein, such as the colon and proteus bacilli. The presence of carbohydrate in the medium may inhibit indol production. The indol is not broken up by these organisms and may be recognized by adding a few drops of a suitable reagent. The tests for indol are described on p. 352.

Special Media. The selective bacteriostatic action of certain anilin dyes is often utilized in the isolation of certain bacteria, which is difficult and uncertain with ordinary media. They are also of some diagnostic value. Among them may be mentioned gentian violet, basic fuchsin, and methylene blue. The specific action of these dyes is not due to their color but to the presence of certain molecular groups which may be colorless. Other substances which exhibit neither color nor fluorescence may have a similar action. Tyrothricin, shown by Dubos (1939) to be produced by *Bacillus brevis,* has an inhibitory effect upon a number of bacteria. When added to culture media in suitable concentrations it facilitates the isolation of such microörganisms as the meningococcus and *Hemophilus influenzae* from nasopharyngeal and throat cultures. Penicillin was first reported in this connection and some other antibiotics have a similar usefulness.

Oxygen Requirement. This is not of much diagnostic value since most pathogens are facultative anaerobes. The obligate anaerobes include the bacilli of tetanus, botulism, malignant edema, and gas gangrene, the treponemata and leptospirae. For special methods of anaerobic cultivation, see p. 918. An important point in the recognition of *Brucella abortus* is its inability to grow on isolation except in an atmosphere of 5 to 10 per cent carbon dioxide.

Serologic Reactions. The complete identification of some species of bacteria, such as the group of dysentery bacilli, or the types of bacteria within a species, such as the pneumococcus, depends upon serologic reactions. This method depends upon the fact that when bacteria are injected into a suitable animal the serum of the animal acquires the property of reacting specifically in various ways with the species of organism injected but not, as a rule, with other species. This property depends upon the development in the inoculated animal of specific protective substances or antibodies. The more important manifestations of this property are: (1) Agglutination of the organisms, (2) precipitation as a result of combination with soluble products of the organism in fluid media, (3) stimulation of phagocytosis of the organisms in the presence of leukocytes, (4) fixation of complement when the organisms or their products are mixed with their specific sera, (5) bacteriolysis of the organisms, (6) neutralization of toxins produced by the bacteria, (7) ability to protect animals from otherwise fatal inoculations of the bacteria due to one or more of these properties and possibly other unknown activities.

While these activities are, in general, specifically limited to the species of bacteria used, they may be exerted to a greater or lesser extent upon biologically related species (group reactions). These group reactions are usually quantitatively less marked and, therefore,

evident only in higher concentrations of the serum than with the specific organism. For example, the serum of an animal inoculated with typhoid bacilli may also agglutinate, to a less extent, paratyphoid bacilli. Occasionally, however, the difference in the degree of the serum action on the two species is slight, and it may be necessary to resort to agglutinin absorption tests or other special procedures (see Chapter 10). These procedures are further complicated by the changes in the antigenic properties of some of the organisms due to dissociation. These special individual changes will be discussed in the chapters dealing with those organisms in which such changes have been studied.

Conversely, the behavior of the serum of an individual with an infectious disease may be tested, similarly, with known species of bacteria or their products in order to determine the etiology of the infection; for instance, the agglutination reactions in typhoid fever and brucellosis.

Cultivation of Viruses

Viruses and rickettsiae require the presence of living cells. Many types of tissue cultures have been devised. The most practical is the use of the fertile egg of a hen.

Cultivation in Chick Embryos. This valuable technic, though used chiefly for the propagation of viruses and rickettsiae, may be and is used for bacteria also. As originally developed by Goodpasture it consists in the inoculation of the chorioallantoic membrane of the embryo in the developing egg which has been incubated at 38° C. for 7 to 10 days. These eggs are "candled" to locate the embryos, and to determine their viability. The location is marked on each egg and a small square or triangle is cut through the shell over the embryo, usually with a dental drill, care being taken not to cut the shell membrane beneath. This piece of shell is lifted off. The shell membrane is then pierced and the inoculum dropped on the chorioallantoic membrane lying beneath. The window in the shell is closed with a sterile coverslip and paraffin, and the egg returned to the incubator for the length of time required to kill the embryo or until there is evidence that the membrane may be infected. This period varies from 2 to 13 days depending on the virus or organism used. Some viruses produce characteristic changes in the membrane.

Various modifications of this original technic have been developed, inoculation being made into the allantoic cavity, the amniotic cavity, the yolk, the embryo itself, and even into the blood stream or brain. Often no window is cut, but the injection made directly through the shell with a hypodermic needle. The yolk sac method developed by Cox has become very popular, especially in the culture of rickettsiae for vaccines. Aseptic precautions are taken throughout these procedures.

Role of Domesticated Animals in the Transmission of Disease

As a rule, disease transfer from domesticated animals is of minor importance when contrasted with the diseases communicated by man to man. In fact, one of the extraordinary findings of immunology is the lack of susceptibility of one kind of animal to the diseases of other species. This resistance even extends to certain varieties of a species.

Table 1 summarizes the diseases contracted by man from domesticated animals.

Animal Experimentation

Animal inoculation is extremely important as a means of isolating and identifying many pathogenic organisms. The susceptibility of a certain species of animal and the type of disease produced is frequently essential in diagnosis. It is a valuable means of isolating and identifying the filtrable viruses and of determining the presence of antiviral antibodies.

Table 1

DISEASES OF ANIMALS TRANSMISSIBLE TO MAN

Disease	*Animal Source*	*Remarks*
Actinomycosis (See p. 89)	Cattle (lumpy jaw)	*Actinomyces bovis* common type. Evidence of human infection from cattle insufficient. Present in and on carious teeth and in tonsillar granules of man
Anthrax (See p. 59)	Cattle, horses, sheep	In cattle, hides; in horses, hair for shaving brushes; in sheep, the wool. *Bacillus anthracis*
Brucellosis (See p. 108)	Goats, cattle, swine	Dairy products main source of infection from cows and goats. Carcass of infected hog at abattoir. Handling of animals by farmers and veterinarians. Bovine strain least virulent *Brucella melitensis, B. abortus, B. suis*
Coccidioidomycosis (See p. 234)	Wild rodents, dogs	Occurs in cattle and sheep as local lymphadenitis, not communicable. *Coccidioides immitis.* Usually acquired by inhalation
Equine encephalomyelitis Eastern, Western, Venezuelan forms. Also St. Louis and Japanese B types (See p. 200)	Birds, horses	Birds probably chief source. Horse may be incidental. Swine, goats, dogs, and sheep susceptible. Mosquito vector. Virus agent
Erysipeloid (Rotlauf) (See p. 98) (Fish handler's disease)	Swine Fish	Contact with bone or carcass of hog with swine erysipelas, or with infected fish. A severe dermatitis, occasionally generalized. Due to *Erysipelothrix rhusiopathiae*
Flukes (See pp. 571, 572)	Cats, dogs, sheep, cattle, and other animals	Cats and dogs—*Opisthorchis felineus* and *Clonorchis sinensis.* Sheep and cattle—*Fasciola hepatica. Schistosoma japonicum* in all
Foot and mouth disease (Aphthous fever) (See p. 197)	Cattle, hogs, sheep, goats	Milk or saliva of infected cow usual cause of occasional human infection. Symptoms in man very mild. Virus agent
Glanders (See p. 120)	Horses	In man an acute and chronic type of disease usually fatal. Acute resembles pyemia; chronic, ulcers and lymphatic gland involvement. *M. mallei*
Hydatid disease (See p. 590)	Dogs	Ingestion of *Echinococcus granulosus* ova. Close association with infected dogs makes possible ingestion of dog fecal material
Jungle yellow fever (See p. 221)	Unknown	Mosquito vector. Virus agent

Table 1—(Continued)

DISEASES OF ANIMALS TRANSMISSIBLE TO MAN

Disease	*Animal Source*	*Remarks*
Leptospirosis (See pp. 157, 158)		
1. Weil's disease	Rats	Ingestion of material containing *Leptospira icterohaemorrhagiae*
2. Canicola fever	Dogs	Ingestion of material containing *L. canicola*
3. Other leptospiroses	Certain rodents	Contact with contaminated materials containing other *Leptospira*
Louping ill	Sheep primarily	Transmitted to man by tick. Found chiefly in Scotland and England. Related to Russian spring-summer encephalitis. Virus agent
Lymphocytic choriomeningitis	Mice, possibly dogs	Virus agent
Milk sickness (See p. 938)	Cows	Milk from cows which have fed on white snake root or rayless golden rod
Plague (See p. 116)	Rats and other rodents	During plague epizootic in rats, rat fleas take up plague bacilli in rat's blood. Feeding on man transfers infection. *Pasteurella pestis*
Psittacosis (See p. 214) Ornithosis	Parrots, parrakeets, love birds Pigeons, chickens, and possibly other birds	Virus disease. Diarrhea in birds, pneumonia in man
Rabies (See p. 206)	Dogs, cats, foxes, skunks, vampire bats	Bite of rabid animal. Vampire bat a healthy carrier. Virus agent
Rat-bite fever (See pp. 147 and 160)	Rats and other rodents	*Streptobacillus moniliformis* or *Spirillum minus*
Rift-Valley fever	Lambs	Contact with lambs sick with this virus disease may produce a dengue-like disease in man
Ringworm	Cats, dogs, horses, cattle, mice	*Microsporum canis* and *M. felineum* frequent in children—scalp and glabrous skin. *Trichophyton mentagrophytes* and *Microsporum gypseum* chiefly from horses. *T. faviforme* from cattle
Salmonella (food poisoning) (See p. 131)	Cattle, rats, mice	Food poisoning from meat of infected cattle or swine, or contamination of food by feces of infected rats or mice
Septic sore throat (See p. 35)	Cows	Human streptococcus (beta type of hemolysis; Lancefield group A) infects udder and milk. Streptococcus of chronic mastitis of cow is an alpha or alpha prime type

Table 1—(Continued)

DISEASES OF ANIMALS TRANSMISSIBLE TO MAN

Disease	*Animal Source*	*Remarks*
Tapeworms (See p. 584)	Cattle, hogs, dogs, rats, and mice	*Taenia saginata*—cattle, *Taenia solium*—hog, *Dipylidium caninum*—dog, *Diphyllobothrium latum*—fish. *Hymenolepis nana* and *H. diminuta*—rats and mice
Trichinosis (See p. 612)	Hogs, rats	Ingestion of raw or insufficiently cooked infected pork meat. Rats eat infected pork meat. Hogs eat garbage containing pork scraps. *Trichinella spiralis*
Tuberculosis (See p. 80)	Cattle and other animals	Bovine tuberculosis most important form contracted from animals by man. Commonest manifestation is some form of bone or joint disease. Can cause pulmonary disease also. *Mycobacterium, tuberculosis,* var. *bovis*
Tularemia (See p. 113)	Wild rabbits, hares, and other rodents	Deer fly or tick transmission. Inoculation from handling infected rabbits. Eating insufficiently cooked rabbit. *Pasteurella tularensis*
Typhus-like fevers		
1. Endemic typhus	1. Rats	1. *Rickettsia mooseri.* Transmitted by rat flea
2. Rocky Mountain spotted fever	2. Wild rodents	2. *R. rickettsi.* Transmitted by wood tick
3. Tsutsugamushi ("scrub or mite typhus")	3. Rats	3. *R. nipponica.* Transmitted by mite
4. Boutonneuse fever	4. Dogs	4. *R. conori.* Transmitted by tick
5. "Q" fever (See p. 176)	5. Bandicoot and possibly other animals	5. Transmitted by tick

The experimental animals most frequently employed are, in the order of their usefulness: the white mouse, the rabbit, the guinea pig, and the white rat. Hamsters are becoming increasingly popular in the study of virus diseases and the leptospiroses. Monkeys are invaluable for some purposes but are too expensive for routine work. Other animals are required for special purposes, such as the ferret for the virus of influenza and kittens for staphylococcus food poisoning. Horses, goats, and rabbits are used for the preparation of therapeutic sera.

Methods of Inoculation: 1. CUTANEOUS. The material is rubbed thoroughly over a shaven area with a glass rod.

2. INTRACUTANEOUS. When local reactions are to be observed white areas of skin are preferable. The hair is removed and the skin disinfected. A small syringe and a sharp needle, preferably No. 26, are used. The needle is inserted, lumen up, into the skin, and lifted slightly before injection. The amount given is usually 0.1 ml. A small, pale wheal should be present immediately after injection.

3. SUBCUTANEOUS. After clipping the hair and disinfecting the skin the material is emulsified and injected with a syringe and hypodermic needle of suitable size. Tissue

may be placed in a pocket made by incising the skin and stripping back the adjacent subcutaneous tissues. Such an incision should be sealed with collodion.

4. Intravenous. In rabbits the marginal ear vein is used. This can be distended by applying either hot water or xylol. In mice a vein at the base of the tail is used and in rats the saphenous vein in the leg. In guinea pigs it is usual to expose the jugular vein or a vein in the leg or to make the injection directly into the heart under anesthesia. In the latter case the needle is inserted in the second right interspace near the sternum, with the tip directed backward, downward, and toward the left. Moderate suction is exerted with the syringe and the depth to which the needle is inserted is varied until blood enters the syringe freely. Injection is then accomplished. The apex beat may be located by palpation and the needle plunged directly into the ventricle at this point.

5. Intraperitoneal. After shaving or clipping and disinfecting the skin of the abdomen the needle is introduced obliquely through the skin in the median line and then vertically through the muscle until the tip is within the peritoneal cavity. To avoid puncturing the gut it is desirable (particularly with rabbits) to hold the animal with the head down so that the intestines gravitate away from the needle.

6. Intramuscular. These injections are usually made into the posterior muscles of the thigh.

7. Intracerebral. *Rabbits.* The head is shaved and the skin sterilized. Under ether anesthesia the skull is exposed by an incision midway between the eye and the medial ridge at the junction of the occipital and parietal bones. A small trephine opening is made and the injection carried out by means of a 1-ml. syringe with a ¼-inch needle. *Mice.* Under ether anesthesia a fine-bore needle attached to a 1-ml. syringe is forced through the skull at a point just anterior and lateral to the vertex, to a depth of about ⅛ inch. The maximum volume to be injected is 1/20 ml.

8. Intratesticular. *Rabbits and Guinea Pigs.* The skin is disinfected, the testicle grasped, a fine needle inserted, and a small amount of the material injected.

9. Inhalation. *Guinea Pig.* The animal is placed in the chamber in which the dust or spray is injected, and allowed to breathe it over a period of time. Or a glass chamber is fitted over the animal's head and an atomizer at the other end of the chamber given one or more puffs with the bulb.

10. Ingestion. Material may be mixed with the food or placed in the animal's mouth with a rubber-tipped pipet or dropper, or may be injected directly into the stomach through a catheter. A soft rubber urethral catheter is suitable for rabbits. A silver Eustachian-tube catheter is useful for mice.

Natural Infections of Laboratory Animals

In human autopsies we appreciate the importance of great experience in recognizing the pathologic lesions caused by the various diseases affecting man. In the study of the lesions in experimental animals experience is equally important. It is essential to be familiar with the appearance of the normal organs of such animals, and with the changes in the tissues and body fluids caused not only by the infection with which the animal has been inoculated, but also those diseases which may occur spontaneously in the animal. In view of the rigid controls demanded in other fields of research, it is remarkable that relatively so little attention has been given to this matter.

Mice. "Mouse typhoid," due usually to *Salmonella typhimurium* or *S. enteritidis,* is an important cause of fatal epizootics in laboratory-bred animals. Hemorrhagic septicemia due to *Pasteurella muricida* (*B. murisepticus*) also occurs. *Streptobacillus moniliformis,* causative agent of a form of rat-bite fever in man, also infects mice. There are other infectious diseases of mice met with less commonly. Tumors—especially adenocarcinoma of the breast—are not uncommon. Sarcosporidiosis, ringworm, and helminthic infections (larval and adult) also occur.

Virus Diseases. Marschal's infectious ectromelia is characterized by cutaneous infections

Table 2

DIAGNOSIS OF INFECTIOUS DISEASES BY ANIMAL INOCULATION

Disease	*Material*	*Animal*	*Method of Inoculation*	*Period of Incubation*	*Pathologic Lesions*	*Septicemia*	*Remarks*
Anthrax	Material from the pustule (blood in septicemia, sputum in pulmonary form)	Mouse, guinea pig, rabbit	S.c.	1 to 2 days	Gelatinous exudate at site of inoculation	Extreme, blood almost black	Bacilli particularly numerous in the spleen
Botulism	Emulsion or filtered extract of suspected food. Filtered culture from food	Mouse, guinea pig	S.c. or orally	1 to 4 days. Possibly 6 days if fed	Death due to cardiac or respiratory failure. May show oculomotor paralyses and mydriasis	O	Inoculate a control animal together with antitoxin
Brucellosis	Blood, urine or material from local focus	Guinea pig	S.c. or I.p.	1 to 3 months	Caseous nodules in spleen, liver, lymph glands. Often caseous epididymitis in males or abortion in pregnant females. Arthritis	+	Animals less susceptible to bovine type. Agglutinins may be demonstrable
Cholera	Culture	Guinea pig	I.p.	1 to 2 days	Peritonitis	O	To identify vibrio inoculate a control animal, together with anticholera serum. Examine the peritoneal exudate for bacteriolysis at 30-minute intervals (Pfeiffer's phenomenon). Not often done

Diphtheria	Broth culture or filtrate (2 ml., 48 hr.)	Guinea pig	S.c.	2 to 4 days	Local edematous swelling with enlarged regional glands. Pleural and peritoneal hemorrhagic effusions. Enlarged hemorrhagic adrenals	O	Inoculate a control animal together with 500 units of antitoxin. Virulence may also be tested intradermally (p. 93) in guinea pigs or rabbits. 10-day chicks may be inoculated S.c.
Friedländer pneumonia	Sputum	Mouse	S.c. or I.p.	1 to 2 days	Peritoneal exudate very sticky	+	Rabbits and guinea pigs are refractory to subcutaneous injection
Gas gangrene	Material from wound; or broth culture	Guinea pig	I.m.	1 to 2 days	Pathogenicity varies. Inflammation and necrosis of tissue with gas formation	±	Differentiate species by control animals receiving also specific antitoxic sera
Glanders	Material from lesion	Male guinea pig	I.p. or S.c. if contaminated	2 to 3 days	Generalized granulomas. Early orchitis from which organisms can be isolated (Straus reaction)	Rare	Consider *Malleomyces pseudomallei* as well as *M. mallei* (glanders bacillus)
Lobar pneumonia	Sputum. Material from lung puncture. Blood	Mouse	I.p. or S.c.	2 hr. to 4 days	General infection with peritonitis	+	For technic of type determinations, etc. see p. 43
Plague	Material from bubo, sputum, blood	Mouse, rat, guinea pig	Cut., S.c.	2 to 5 days	Hemorrhagic lymphadenitis. Caseous areas in glands, spleen, liver, occasionally other organs. Characteristic lesions in lungs of guinea pigs	+	The ability to infect through the unbroken skin is of some value in diagnosis
Staphylococcus food poisoning	Food or broth culture	Kitten	Oral	1 hr.	Vomiting	O	Due to a preformed toxin. Not an infection
Tetanus	Material from wound; preferably filtrate from broth culture	Rat, mouse, guinea pig	S.c.	1 to 5 days	Tetanus. Spasticity begins in muscles nearest point of inoculation	O	Inoculate a control animal and give a prophylactic dose of antitetanic serum. Rats show typical "seal gait"

Table 2—(Continued)

DIAGNOSIS OF INFECTIOUS DISEASES BY ANIMAL INOCULATION

Disease	*Material*	*Animal*	*Method of Inoculation*	*Period of Incubation*	*Pathologic Lesions*	*Septicemia*	*Remarks*
Tuberculosis	Sputum, urine, spinal fluid, exudates, etc.	Guinea pig	S.c.	4 to 8 wks.	Local caseous lesions with involvement of regional, and later other lymph glands. Tubercles in spleen, liver and other organs	O	If material is badly contaminated, treat with alkali, wash and neutralize before injection
Tularemia	Material from primary lesion, lymph nodes, or blood. Sputum	Mouse, guinea pig, rabbit	S.c., I.p. scarification	2 to 7 days	Hemorrhagic lymphadenitis. Caseous areas in glands, spleen, liver, and occasionally in other organs	+	Several animals should be injected
Relapsing fevers	Blood	Mouse, rat	I.p.	2 to 5 days	Organisms appear in blood after 1 to 2 days and persist for 2 to 4 days	+	Caused by several species of genus *Borrelia*, immunologically different. Similar clinical picture. Relapses may occur in mice. Infection never fatal
Leptospirosis 1. Weil's disease 2. Canicola fever 3. Other leptospiroses	Blood, urine	Hamster, mouse, guinea pig	I.p.	3 to 12 days	Jaundice, hemorrhages in lungs, serous cavities, muscles, etc.	+	Leptospiras can be demonstrated in blood and tissues. 1. Fatal for hamster, mouse, and guinea pig 2. Mouse—immune, guinea pig—fever and weight loss, fatal for hamster
Rat-bite fever							
1. Due to *Spirillum minus*	Local lesions or regional glands, blood	Mouse, rat, guinea pig	I.p.	5 to 14 days	Examine blood. In guinea pigs pyrexia and enlarged lymph nodes. Alopecia and exudate around eyes	+	Rats and mice develop a sepsis but do not die
2. Due to *Streptobacillus moniliformis*	Same	Mouse	I.p. or S.c. in tail or foot pad	I.p. 72 hr. S.c. 3 to 7 days	Lesions in liver and spleen (I.p.) Multiple transitory arthritis (S.c.)	+	Fatal for mice

Epidemic typhus	Blood 1 ml.	Guinea pig (nearly grown male) Rats	I.p.	After 5 to 12 days, fever lasting 5 to 9 days	No gross scrotal or testicular lesions. Animal recovers	+	Blood and brain infective during fever. After recovery animal is immune
Endemic typhus	Blood 1 ml.	Guinea pig (nearly grown male)	I.p.	After 5 to 12 days, fever lasting 1 to 9 days	Swelling of testicle and erythema of scrotal skin due to inflammation of tunica vaginalis. Animal recovers	+	Same
"Q" fever	Blood	Guinea pig, mouse	I.p.		Fever, large spleen	+	
Rocky Mountain spotted fever	Blood	Guinea pig (nearly grown male)	I.p.	After 2 to 10 days, fever lasting 5 to 15 days	In severe type only, scrotal skin shows thrombosis and necrotic edema leading to gangrene. Mortality 95%. In mild type less marked scrotal lesions. Mortality 40 to 50%	+	Same
Tsutsugamushi disease (scrub "typhus")	Blood	Mouse, guinea pig	I.p.	7 to 14 days	Ruffled fur, peritoneal exudate tenacious and stringy	+	May show no symptoms by 14th day when passage is due
Coccidioidomycosis (coccidioidal granuloma, San Joaquin Valley fever)	Pus, sputum and culture	Male guinea pig, mouse	I.p., I.t.	6 days	Typical lesions showing endogenous sporulation	O	Useful in differentiation from blastomycosis

Table 2—(Continued)

DIAGNOSIS OF INFECTIOUS DISEASES BY ANIMAL INOCULATION

Disease	*Material*	*Animal*	*Method of Inoculation*	*Period of Incubation*	*Pathologic Lesions*	*Septicemia*	*Remarks*
Leishmaniasis	Blood or material aspirated from glands, spleen, marrow, etc.	Chinese hamster, mouse, rat, dog	I.p., large doses	Long and variable	Generalized lesions similar to human disease	O	Inoculation usually unsuccessful. Not a useful diagnostic procedure at present
Trypanosomiasis							
1. African (sleeping sickness)	Blood, gland juice	Rat, guinea pig	I.p.		Examine blood at intervals after about two weeks	+	Gambian and Rhodesian forms
2. American (Chagas' disease)	Blood, viscera	Guinea pig, young rat	I.p.	10 to 14 days	Presence of trypanosomal form in blood, or leishmanian form in heart muscle or viscera	+	Organism has cycle of 2 forms, leishmanian and trypanosomal
Dengue	Blood in first 3 days	Monkeys	S.c.	5 days	Dengue	+	
The encephalitides							
1. Encephalitis, St. Louis type	Brain emulsion (with all types). Blood. Spinal fluid	Mouse (all types)	I.c. (all types)	4 to 7 days	Encephalitis in all types		Serum from cases of Economo type does not protect from these 6 types. Except for No. 6, which is transmitted by a tick, these encephalitides are epidemiologically alike. All are immunologically distinct

2. Japanese B encephalitis				3 to 10 days			
3. Eastern equine encephalomyelitis				2 to 3 days			
4. Western equine encephalomyelitis				5 to 7 days			
5. Venezuelan encephalitis							
6. Spring-summer encephalitis of Russia							
Foot-and-mouth disease	Milk, saliva, excreta; fluid of vesicles, blood	Guinea pig	I.p., S.c. I.d. (foot pads) I.n.	4 days	Typical vesicles	+	Three immunologic types, O, A, and C
Herpes febrilis	Vesicular fluid, saliva, blood, spinal fluid	Rabbit, mouse	Corneal I.c.	4 to 7 days	Keratitis. Sometimes encephalitis. Papular eruptions		Inclusion bodies in corneal epithelium

Table 2—(Continued)

DIAGNOSIS OF INFECTIOUS DISEASES BY ANIMAL INOCULATION

Disease	*Material*	*Animal*	*Method of Inoculation*	*Period of Incubation*	*Pathologic Lesions*	*Septicemia*	*Remarks*
Influenza	Nasopharyngeal washings	Ferret, mouse	I.n.	48 to 72 hr.	Mice often develop pneumonia due to virus alone or secondary invaders. Characteristic consolidation of lung in ferret and mouse	O	Ferret used for isolation. After isolation the virus may usually be readily adapted to the mouse
Louping ill	Tissues of brain and cord	Mouse, rat, sheep	I.c. I.n.		Encephalitis, especially of Purkinje cells of cerebellum	+	Inclusion bodies in nerve cells. Related to Russian spring-summer encephalitis. Primarily a disease of sheep
Lymphocytic choriomeningitis	Brain, spinal fluid, blood	Guinea pig, monkey, mouse	S.c., I.p. I.c., I.n.	5 to 9 days	Typical choriomeningitis. Bronchopneumonia. Sometimes necrotic areas in other organs	+	Chronic lesions may persist after recovery, especially in kidney. Animal becomes a carrier
Lymphogranuloma venereum	Pus from bubo	Mouse, guinea pig	I.c.		Encephalitis		Brain emulsion can be used to prepare Frei antigen
Poliomyelitis	Brain or cord emulsion. Feces	(a) Monkey (b) Cotton rat, mouse	(a) I.c., S.c., I.p., I.n. (b) I.c.	2 days to 2 mos.	Typical disease in monkey	+ Transient in animals	Cotton rat and mouse usually susceptible only to special strains. Virus not present in human C.S.F. Not a diagnostic procedure
Psittacosis	Filtered sputum, blood	Mouse, guinea pig, parrot	I.p.	7 to 10 days	Focal necroses in liver and spleen. Psittacosis bodies in reticuloendothelial cells	+	Liver lesions diagnostic

Rabies	Brain, salivary gland	Mouse, guinea pig, rabbit	I.c. I.m. I.p.	10 to 14 days (mouse). Within 3 wks. (guinea pig, rabbit)	Negri bodies in hippocampus, fissure of Rolando, cerebellum	O	Animal inoculation especially useful if suspected dog is decomposed. Mice may show Negri bodies by 6th or 7th day. With street virus inject 0.03 ml. of 10% brain tissue by weight
Rift-Valley fever	Blood, tissues	Mouse	I.p., S.c. I.n. Cut.	2 to 5 days	Focal necroses in liver with intranuclear inclusion bodies	+	Liver lesions diagnostic. Found chiefly in East Africa
Variola	Material from skin lesions	Rabbit	I.d. Corneal	5 days 2 to 3 days	Local reaction Keratitis		McKinnon's test Paul's test
Yellow fever	Blood (first 3 days), liver, spleen	(a)Mouse (b)Monkey	(a) I.c. or I.p. after I.c. injection of starch (b) I.p.	5 to 10 days	(a)Encephalitis (b)Typical lesions in viscera	+	(a) Protection tests used to detect presence of antibodies in epidemiologic studies (b) Characteristic lesions in the liver with intranuclear inclusions in monkey
Trichinosis	Suspected food	Mouse, rat	Feeding	2 wks.	Examine diaphragm for encysted larvae	+ Transient	Not as much used now as other tests

Cut. = cutaneous
I.c. = intracerebral
I.d. = intradermal
I.n. = intranasal
I.p. = intraperitoneal
S.c. = subcutaneous
I.t. = intratesticular

progressing to gangrene, or by abdominal lesions. Cytoplasmic inclusions may be present. Diseases of the salivary glands, pneumonias, and lymphocytic choriomeningitis due to viruses have been reported. Theiler's disease, which causes a flaccid paralysis of the hind legs, occurs spontaneously.

Guinea Pigs. Disease-free colonies of these animals have been maintained, but they are not common. The diseases found vary with the stock and environment, and workers using guinea pigs extensively should familiarize themselves with those conditions found in the uninoculated animals. The infections most commonly found are those due to certain salmonellas, a parotitis, and a nonhemolytic streptococcus infection of the lymph glands, especially of the neck, described by Theobald Smith. Spontaneous or cage infection with tuberculosis has been observed.

Rabbits. "Snuffles" is probably the most common and most inconvenient ailment. It is due to *Brucella bronchiseptica* (*Bacillus bronchisepticus*) or to *Pasteurella cuniculicida* (*Bacterium lepisepticum*). Rabbits suffer spontaneous infections with pseudotuberculosis, usually due to *Pasteurella pseudotuberculosis*. Diarrhea may be due to coccidiosis, a very common infection in rabbits. Infections occur with some spirochetes, which involve the genitalia and may be confused with *Treponema pallidum* of man.

A spontaneous encephalitis has been reported in Europe and America. Fungus infections are common. Mites may cause skin lesions in and about the ears. The commonest helminthic infection is with the larval stage of *Taenia pisiformis* of the dog. The cysticerci are found in the omentum and liver of infected rabbits. Tumors are rare.

Virus III, described by Rivers, causes an acute orchitis.

Myxomatosis is responsible for severe and highly fatal outbreaks.

Rats. If properly cared for, rats are relatively free from spontaneous diseases, except mange which especially involves the ears and tail. The most common specific infections encountered are due to *Salmonella typhimurium* and *S. enteritidis*. Nonspecific middle-ear infections and pneumonias are fairly common. The liver may show the larval stage of the cat tapeworm.

Monkeys. Monkeys are susceptible to tuberculosis which often spreads through a colony. They are susceptible to some forms of bacillary dysentery (*Shigella paradysenteriae*, Flexner). A lung mite produces symptomless lung lesions which may prove confusing unless understood.

Fowls. Chickens may develop "fowl diphtheria" or a laryngeal inflammation as in fowl pox. They are also susceptible to an epidemic diarrheal disease due to *Salmonella pullorum*.

Collecting Material at Biopsy or Necropsy of Man or Animals

It is customary to sear with a heated knife or spatula a spot on the surface of the organ from which cultures are to be made. Then a sterile platinum spud is introduced at this point, and tubes of culture media are inoculated with the spud. The platinum loop may be used when an incision has been made into the organ with a sterile knife.

A capillary pipet is a good instrument for taking up blood from the right side of the heart or from blood vessels. When great precaution is necessary to insure sterilization of the surface, as in cultures of an excised gland or organ, the piece of tissue may be dropped into 5 per cent formalin solution for a few minutes, washed in sterile salt solution, next placed in a sterile Petri dish and the material obtained from the center; or it may be dropped for a few seconds into boiling water. Before performing a necropsy on experimental animals it is well to dip the dead animal into 3 per cent trikresol solution.

In autopsying small laboratory animals they may be tacked down by each foot to a wooden board covered with heavy paper, the paper with the animal being thrown in the furnace after the autopsy. When pans are used these should be put in the autoclave. It is advisable to use rubber gloves when autopsying animals infected with virulent organisms. To avoid conjunctival infection it is well to wear goggles or a suitable mask.

CHAPTER 2

Cocci

Key and Notes

Streptococcus Forms. Cells in short or long chains; never in packets.

I. Aerobic

A. Pyogenic group. These produce a zone of *beta* (clear) hemolysis around colonies on blood agar.

1. Lancefield's group A (primarily pathogenic for man).
   a. *Streptococcus pyogenes.*
   b. Other unnamed or less well-recognized species.
2. Lancefield's group C.
   a. "Animal pyogenes."
   b. *Streptococcus equi* (causes "strangles" in horses).
   c. *Streptococcus equisimilis* (may infect either man or animals).
3. Lancefield's group B.
   a. *Streptococcus agalactiae* (causes mastitis in cattle) (*S. mastitidis*).

B. Viridans group. Colonies on blood agar surrounded by a greenish zone of methemoglobin (*alpha* hemolysis).

1. Lactose fermented.
   a. *Streptococcus salivarius.*
   b. *Streptococcus mitis* (*S. viridans*).
   c. *Streptococcus bovis.*
2. Lactose not fermented.
   a. *Streptococcus equinus.*

C. Enterococcus group (Lancefield's group D). May or may not show zone of *beta* (clear) hemolysis around colonies on blood agar. Infects either man or animals.

II. Anaerobic. Usually nonhemolytic and gas-producing. May cause puerperal sepsis.

Diplococcus Forms. Cells usually in pairs. Exacting in media requirements. Oval to lancet-shaped forms, less frequently in chains. Colonies greenish on blood agar with greenish zone of methemoglobin (*alpha* hemolysis). Bile-soluble.

I. *Diplococcus pneumoniae* (Pneumococcus). Seventy-five serologic types have been described. Ten types are responsible for 75 per cent pneumococcus pneumonias. (See text.)

II. Anaerobic species of diplococcus.

Staphylococcus Forms. Cells as a rule in irregular groups. Pigment white or orange, or less commonly lemon-yellow. Placed now in genus *Micrococcus.*

I. Aerobic. Usually ferment lactose and liquefy gelatin.

1. *Micrococcus pyogenes.*
   a. Orange pigment.
      M. pyogenes, var. *aureus* (*Staphylococcus aureus*).
   b. No pigment. White growth on solid media.
      M. pyogenes, var. *albus* (*Staphylococcus albus*).
   c. Lemon-yellow pigment.
      M. citreus (*Staphylococcus citreus*).

II. Anaerobic. Several less known species.

Tetrad Forms. Cells in tetrads, often surrounded by a capsule in the animal body.

1. *Gaffkya tetragena* (*Micrococcus tetragenus*).

Sarcina Forms. Division occurs in three planes, producing regular packets. Growth on agar abundant, usually with the formation of yellow or orange pigment.

A. Yellow pigment formed.

Gelatin not liquefied.

1. *Sarcina ventriculi* (stomach contents of man and animals).

Gelatin liquefied.

2. *Sarcina flava* (air).
3. *Sarcina lutea* (air, soil, water).

B. Orange pigment formed.

4. *Sarcina aurantiaca* (air, water).

Gram-negative Cocci. Neisseria. Cells normally in pairs with adjacent sides flattened.

A. Grow best at 35° to 37° C. on special culture media containing blood, blood serum, or starch, or on infusion agar with vitamin.

1. *Neisseria gonorrhoeae* (Gonococcus).
2. *Neisseria meningitidis* (*N. intracellularis*) (Meningococcus). Four serologic groups: I, II, II_{alpha}, IV.

B. Grow well at 22° C. on ordinary culture media.

Nonchromogenic.

3. *Neisseria catarrhalis.*
4. *Neisseria sicca.*

Chromogenic. Golden or greenish-yellow pigment.

5. *Neisseria perflava.*
6. *Neisseria flava.*
7. *Neisseria subflava.*
8. *Neisseria flavescens.*

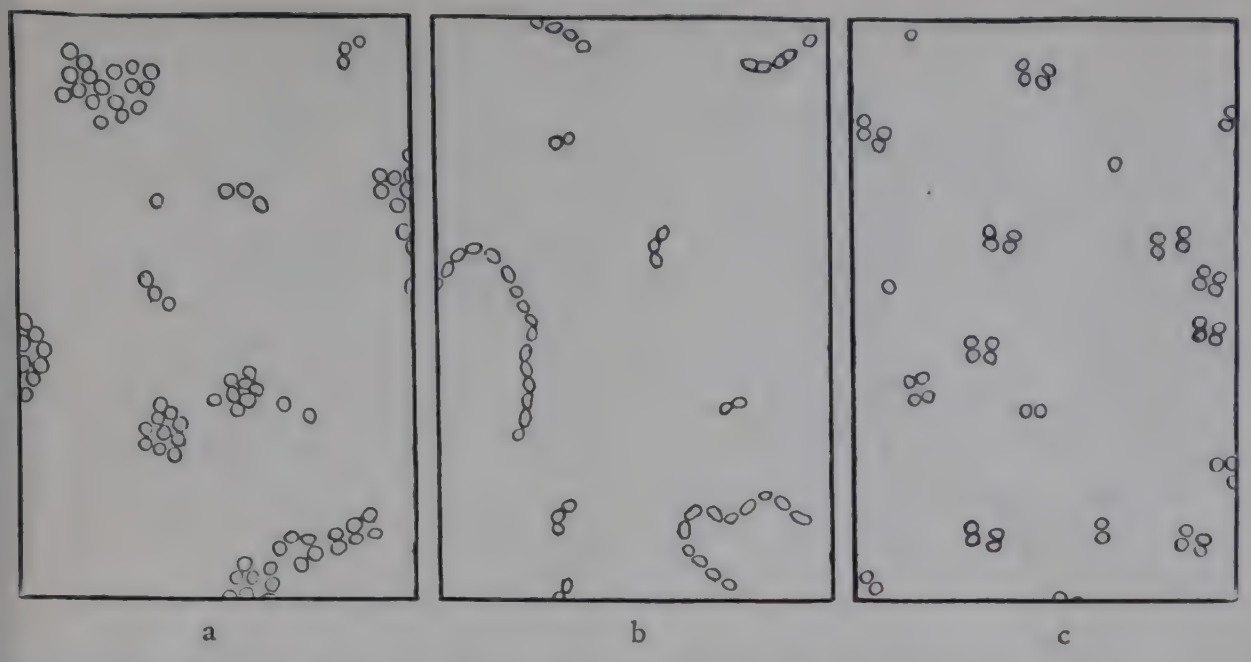
Gram-negative Cocci. Veillonella. Cells in clumps resembling minute staphylococci. Strict anaerobes. Harmless parasites found in mouth and digestive tract of man and animals.

1. *V. parvula.*
2. *V. branhamii.*
3. *V. gazogenes.*

Streptococcus Group

Occurrence. Streptococci are widely distributed in nature and are the cause of a great variety of infections, both in man and animals. They are frequently found in the normal mouth, nose, and intestinal tract, and also in water, milk, and dust. Many are harmless and some of them perform useful functions. On account of the heterogeneity of their activity, their study is very important, and the presence in cultures, particularly of those from the respiratory tract, must be interpreted with great care.

Morphology. Streptococci are typically round. Their multiplication in one plane only results in the formation of chains which may be long or short. They often occur as diplococci in blood media, and sometimes on solid media as well, but in fluid media the chain formation is striking. They are usually Gram-positive. There is an extraordinary difference in size in various strains, and many of them, especially in old cultures, show bizarre, elongated, or club-shaped forms. Occasionally capsules can be demonstrated on pathogenic strains.



Arrangement of cocci. Drawing, Naval Medical School. (a) Staphylococci. (b) Streptococci. (c) Diplococci.

Cultural Characteristics. Most streptococci are aerobic. Some, however, are strictly anaerobic and others require a partial oxygen tension. They grow best at about 37° C. on media enriched with blood or serum. On agar plates the surface colonies are small, grayish, and round, while deep colonies are lenticular in shape. The appearance of the colonies on blood agar (free from carbohydrates) is quite characteristic, and forms the basis for the differentiation into the following broad groups (Schottmueller, Brown):

1. Hemolytic group (beta type). Colonies surrounded by a clear zone of hemolysis.
2. Viridans group (alpha type). Colonies green with a surrounding greenish discoloration of the blood agar, owing, presumably, to the formation of hydrogen peroxide and methemoglobin.
3. Nonhemolytic group (gamma type). Colonies grayish. No change in the surrounding medium.

In broth many streptococci tend to form a flocculent growth which adheres to the side of the tube, and after three or four days falls to the bottom. This tendency to flocculation causes difficulty in evaluating the results of agglutination tests. Frequent transfers on special media may be necessary to minimize this spontaneous clumping but this may result in a change in the characteristics of the cultures. The optimum pH for cultivating pathogenic streptococci is 7.4 to 7.6, and the most favorable temperature is approximately 37° C. Some freshly isolated strains will not grow unless the medium is enriched with serum or blood. Addition of a small amount of glucose (0.1 to 0.5 per cent) to an ordinary meat infusion medium may facilitate growth of such strains. Milk is acidified and usually coagulated.

Many of the carbohydrates are fermented, generally without gas formation. In testing these reactions it is necessary to add the various sugars to meat infusion broth (or agar medium) which has been made sugar free. The addition of an indicator such as bromthymol blue to the medium facilitates the reading of the reaction. Species differentiation of streptococci has been attempted by testing their action on lactose, mannitol, and salicin. Correlation of these biochemic activities with serologic reactions will be discussed under the heading *Classification and Biologic Properties*. As a rule pathogenic strains ferment lactose and salicin, and the enterococci ferment mannitol. Streptococci do not ferment inulin, though a few nonhemolytic strains are exceptions to this rule.

Differentiation from the Pneumococcus. The most important criterion in differentiating streptococci from pneumococci is the solubility of the pneumococcus in bile. To carry

out this test, to 2 ml. of a 24-hour broth culture of the organism about 0.1 ml. of bile (or a 10 per cent solution of sodium taurocholate) is added. The mixture should remain in the incubator for one-half hour. Streptococcus cultures remain unchanged, whereas the turbidity of the pneumococcus culture clears up. The solution can also be observed in a hanging-drop preparation. Control tubes with known bile-soluble and non-bile-soluble cultures should be included.

Variation. Todd has described dissociative changes in hemolytic streptococci after prolonged cultivation on artificial media. When freshly isolated the colonies are "matt-surfaced," later they become smooth and glossy. This change is accompanied by a permanent loss of virulence, although the production of the hemolysin is not affected. The "matt" colonies appear "rough" (R) and the "glossy" colonies appear "smooth" (S). In streptococci, virulence is usually associated with the "matt" form. Recently Loewenthal has reported that the matt-surfaced colonies are changed by mouse passage into a mucoid form as the virulence of the streptococcus for mice is enhanced.

Classification and Biologic Properties

For well over 50 years the classification of streptococci has been a challenge to bacteriologists. The problem has been approached from many angles. Separation of streptococci into three groups on the basis of reaction on blood agar (Schottmueller, Brown), as described on pp. 31–33, has given a practical working basis for many years. Differentiation by fermentation has been found to be of secondary importance. The reaction of streptococci to various types of bacteriophage (Lancefield, Evans) has an interesting significance when considered in the light of other findings.

Agglutination studies with streptococci have been complicated both by a marked tendency to spontaneous clumping and by cross reactions. However, by a special slide agglutination technic Griffith identified 27 types of hemolytic streptococci.

More recently Lancefield has used extracts of massive cultures of streptococci in precipitation tests with monovalent rabbit sera. She found that streptococci fall into fairly distinct groups, each group possessing a specific antigenic substance which differs chemically from the specific antigenic substance of other groups. Other specific substances for distinguishing types within the groups have been demonstrated by a similar technic. Lancefield's classification of the beta hemolytic streptococci has been widely confirmed and 11 groups have been described. These groups are designated by capital Arabic letters which must not be confused with the Greek letters used by Brown to denote the type of hemolysis.

It is interesting to note that by means of Lancefield's groups other classifications of streptococci become correlated, and that the groups have epidemiologic significance. The groups of medical importance are as follows:

Group A includes most of the beta hemolytic streptococci that are primarily pathogenic for man. *Streptococcus pyogenes,* the typical species, is probably responsible for a larger list of diseases than any other microörganism. All strains yield the group-specific C (carbohydrate) substance which is the characteristic component of this group. Griffith's types within group A can be distinguished by precipitin tests with the M substance, from *matt* variants, which is protein in nature. Type specificity, M substance, and virulence are associated in streptococci. A "T" substance, also associated with type, may be found in both matt and glossy strains.

Group A streptococci ferment trehalose as well as lactose and salicin, but not mannitol nor sorbitol. They are resistant to filtered "Phage B," but sensitive to the nascent phage. In addition to hemolysin, they may produce leukocidin, human fibrinolysin, and the erythrogenic toxin which causes the characteristic rash in scarlet fever.

Streptococci of group B also produce beta hemolysis. These microörganisms are primarily bovine in origin. Their group-specific substance is also a carbohydrate, but chemically different from the C substance of group A. In group B the type-specific antigen,

called the "S" substance, is likewise a carbohydrate. Strains of this group will hydrolyze sodium hippurate and are sensitive to Phage D.

Group C, also comprised of beta hemolytic streptococci, includes strains pathogenic for both animals and man. The human strains ferment trehalose but not sorbitol, produce human fibrinolysin, and are sensitive to Phage B filtrate. The group-specific and type-specific substances are different from those of groups A and B.

Group D contains the enterococci which may or may not be beta hemolytic. Mannitol is fermented by strains of this group. Characteristic group-specific and type-specific substances are obtained here also.

No group-specific antigens have been obtained from the viridans group or from the anaerobic group of streptococci.

Susceptibility to Drugs and to Antibiotics. Sulfanilamide (para-amino-benzene-sulfonamide) and many of its derivatives exert a bacteriostatic effect on the growth of hemolytic streptococci *in vitro,* and also a protective and curative action in most hemolytic streptococcus infections. Hemolytic streptococci are also sensitive to penicillin as a rule. Enterococci and some members of the viridans group are resistant to these agents. Other viridans strains are moderately susceptible to penicillin.

Hemolytic Streptococcus Infections

Most of the hemolytic streptococci which cause disease in man are of group A, although some are of group C. They cause inflammations which are diffuse and phlegmonous in character with tissue necrosis rather than pus formation, whereas the staphylococci, as a rule, produce circumscribed, purulent lesions. The ordinary follicular tonsillitis is frequently caused by streptococci of this type, and severe epidemics of sore throat have been spread by contaminated milk from cows whose udders were infected by human carriers of these streptococci. Erysipelas is caused by streptococci of this group. These microörganisms are frequently secondary invaders in diphtheria, tuberculosis, smallpox, and even in typhoid fever, and are the most common cause of the severe bronchopneumonias which may follow the acute infectious diseases, particularly measles and influenza.

Hemolytic streptococci are frequently the cause of wound infections, of otitis media, mastoiditis, and sinus thrombosis, and of puerperal infections. A rapidly fatal septicemia with or without a meningitis may result from any of these conditions. Hemolytic streptococci of other groups cause various diseases peculiar to animals, such as "strangles" in the horse (*S. equi* of group C), and contagious mammitis in cows (*S. agalactiae* of group B).

SCARLET FEVER

As early as 1886 Klein gave the name *Streptococcus scarlatinae* to those strains that he found in the throats of scarlet fever patients. Evidence associating streptococci with this disease accumulated, and in 1923 the Dicks produced typical scarlet fever in volunteers with their *S. scarlatinae* which they considered specific in causing it. Later studies during the ensuing years have shown that a number of Griffith's types of Lancefield's group A can be associated with scarlet fever. All of these produce an erythrogenic toxin which is responsible for the rash as-

sociated with this infection. Although many workers consider *S. scarlatinae* as a definite species, it is generally included in *S. pyogenes.*

The toxin can be demonstrated in Berkefeld filtrates of broth cultures by injection intradermally into susceptible individuals, producing a characteristic skin reaction in them. The toxin is standardized by determining the minimum amount which, when injected intracutaneously, will induce a certain standard reaction.

The Dick Test. This test is used to determine susceptibility to scarlet fever and is carried out in the same manner as the Schick test for diphtheria. Over 80 per cent of adults give a negative reaction to the toxin and have, therefore, a natural antitoxin (and perhaps other antibodies) in the blood. The reaction to the test is usually positive during the early stages of scarlet fever and gradually becomes negative during convalescence.

A saline dilution of the standard toxin with a potency of one skin-test dose in 0.1 ml. is used. This is injected intradermally in the flexor surface of the forearm. Positive reactions appear in from 4 to 12 hours and are read within 24 hours, preferably in the eighteenth hour. At the height of the positive reaction there is a circumscribed area of redness and infiltration varying from 1 to 3 or 4 cm. in diameter according to the susceptibility of the individual. A reaction which has entirely faded in 24 hours is negative. Pseudoreactions are rare in children and controls are not necessary.

Immunization. An active immunity can be produced by four or five subcutaneous injections of increasing doses of the toxin at weekly intervals (Gabritschewsky, the Dicks). The most recently adopted dosage for this purpose is: (1) 650 S.T.D.; (2) 2500 S.T.D.; (3) 10,000 S.T.D.; (4) 30,000 S.T.D.; (5) 100,000 to 120,000 S.T.D. Severe reactions may occur. Three weeks after the last injection the presence of antitoxin is ascertained by another Dick test.

Immunity to the toxin is more easily acquired and more lasting than the immunity to the streptococci themselves; consequently persons may contract severe infections due to these strains of streptococci without developing a rash. In fact, septic sore throat may be the same disease without the rash and the subsequent desquamation.

Schultz-Charlton Reaction. Antiscarlatinal serum or convalescent serum (0.1 to 0.2 ml.) injected intradermally in an erythematous area will cause a definite and permanent blanching of the surrounding scarlatinal rash within five or six hours. This phenomenon is due to a local neutralization of the toxin. It occurs only in the scarlet fever exanthem and is, therefore, a useful diagnostic test in doubtful cases.

Serum Treatment. By immunizing horses with toxic filtrates of scarlet fever streptococci an immune serum can be obtained which is used for prophylaxis and treatment. This is standardized by its ability to neutralize a given amount of toxin. One unit is the smallest amount which will neutralize 50 skin-test doses of a standard scarlatinal streptococcus toxin when compared with the standard antitoxin. A Dick-positive individual may be rendered Dick-negative within 24 to 48 hours by a prophylactic dose, but the protection conferred lasts only for from two to four weeks. Moser, Dochez, and later the Dicks, found that immune serum was effective, when given early, in causing a disappearance of the scarlatinal rash and in relieving symptoms. The dosage recommended is from 9000 to 10,000 units, or more in severe cases. Convalescent serum obtained from the fourth to the seventh week has also been used intramuscularly in doses of 50 to 100 ml. Immune serum must be given early to be effective, certainly not later than the fourth day. It appears to be of no use after complications have developed.

Sulfonamide and Penicillin Therapy. Sulfonamides and penicillin are useful in controlling the infection but do not neutralize toxin that has been formed.

RHEUMATIC FEVER AND RHEUMATIC ARTHRITIDES

The pathogenesis of rheumatic fever and of the rheumatic arthritides is unknown. The mechanisms through which group A hemolytic streptococcus infections may take part in the process are correspondingly obscure.

Attacks of rheumatic fever and the onset and exacerbations of some types of arthritis are frequently preceded by acute hemolytic streptococcus infections, often of trivial character. In such instances the development of rheumatic manifestations is associated with no characteristic modification in the pattern of demonstrable circulating immune substances, produced apparently in response to the hemolytic streptococcus infection. The immune substances may include antistreptolysins "O" and "S" (a lowering of the "S" titer is said to characterize the acute stage of rheumatic fever), and antifibrinolysins, as well as agglutinins, precipitins for the type-specific M substance, complement-fixing antibodies, and opsonins.

During rheumatic fever, hemolytic streptococci may or may not be demonstrable in throat, nose, or other focus, and are rarely found in the blood stream. They are found much more frequently during the antecedent infections. Discrimination should be used in ascribing a causal relationship to any strains of hemolytic streptococci isolated from the patient during the usually protracted course of rheumatic disease, as the appearance of such strains may be incidental. A study of such organisms in relation to the circulating antibodies should give information in such a case. No criteria serve to distinguish those strains which appear to be associated with rheumatic disease.

Nevertheless experience has shown that avoidance of streptococcus infections constitutes an effective measure of prophylaxis.

Currently, the hypothesis that rheumatic disease constitutes a hypersensitive reaction to products of hemolytic streptococci is favored. This is based in part on the resemblance of the arterial lesions in rheumatic fever to those in other diseases generally believed to be allergic, such as serum disease and severe reaction to sulfonamides.

GLOMERULAR NEPHRITIS

Indirect evidence of a character similar to that manifest in rheumatic disease indicates that beta hemolytic streptococci are probably the cause of many cases of acute glomerular nephritis. This latter disease is often associated with, or quickly follows, acute infections with these streptococci, particularly scarlet fever and acute follicular tonsillitis. Such patients often have a positive skin reaction to toxic filtrates of hemolytic streptococci and show a high titer of antistreptohemolysins in the blood (Longcope, 1929, 1936). Winkenwerder et al. (1935) found that in the acute cases which have followed an acute infection, recovery was the rule, but when associated with a chronic or recurring infection the nephritis was progressive.

Nonhemolytic and Viridans Streptococcus Infections

Nonhemolytic and viridans streptococci are frequently the cause of infections of the tonsils, sinuses, middle ear, and teeth, and also of the gall-bladder and

appendix. The lesions produced are of a more chronic type than those due to hemolytic streptococci, and are frequently overlooked. The great importance of these infections is the part which they play in the various focal infections. Other organisms may also be concerned, but streptococci are by far the most important etiologic agents.

SUBACUTE BACTERIAL ENDOCARDITIS

This is the most serious disease due to human strains of streptococci other than those causing the beta type of hemolysis. The portal of entry of the organism is presumably some chronic focus of infection, probably most often infected teeth or tonsils. In some cases the endocarditis has started acutely immediately after a tonsillectomy or a tooth extraction. Operative procedures affecting such foci rather regularly provoke transient bacteremia although endocarditis is rarely established. In the latter instances the organisms lodge in the heart valves; individuals who have experienced rheumatic fever are relatively highly susceptible. This susceptibility is probably due to altered immunologic reactivity of the tissues rather than a localization due to previous damage, because relatively sound valve leaflets may be involved in preference to those which show rheumatic lesions. The microörganisms live and multiply in the heart valves in vegetations in which they appear to be protected from the leukocytes and antibacterial substances in the blood. Embolic phenomena are frequent; arthritis may occur, although without suppuration such as may occur in other forms of sepsis. The disease may last for months or even several years, and remissions are not infrequent, but the outcome is almost invariably fatal, unless energetic treatment is undertaken with an appropriate bacteriostatic substance such as penicillin. The organisms are present in the blood stream and may be grown from the blood by the ordinary technic. Growth is usually slow, and colonies appear in the plates as a rule in two or three days, occasionally only after five or six days. During the remissions the bacteria may disappear from the blood, and repeated blood cultures may be necessary to demonstrate them. In the later stages they may be present in the blood in enormous numbers, sometimes many thousand per ml. A positive blood culture, however, is not necessarily serious if the heart is normal. Even in rheumatic individuals, growth of a few streptococci in blood culture does not justify a definite diagnosis of bacterial endocarditis except in the presence of embolic phenomena or other clinical manifestations of the disease. Other organisms (Pfeiffer bacillus, pneumococcus, gonococcus, etc.) may cause a similar clinical picture in about 5 per cent of the cases. This condition is easily differentiated from sepsis and endocarditis due to hemolytic streptococci (occasionally staphylococci and pneumococci) in which the clinical picture is that of a fulminating infection. The origin of the infection (infected wounds, mastoiditis, puerperal sepsis, etc.) is usually evident. Blood cultures determine the type of organism concerned.

Infections Due to Anaerobic Streptococci and Enterococci

Pathogenic anaerobic streptococci have been isolated in a number of conditions, particularly lung abscesses, often in association with the spirochetes and fusiform bacilli of

Vincent. They have also been found in the blood in puerperal sepsis (10 per cent to 40 per cent). Studies made by Colebrook (1930) showed that these strains were serologically heterogeneous, and that some of them produced fetid gas when blood was present in the medium. Several species have been described.

Enterococci are found in the human and animal intestine. They have a high resistance and a wide temperature range of growth; hence they are found in nature under diverse conditions. They fall into Lancefield's group D. Some produce beta hemolysis, and most of them ferment mannitol. Their pathologic significance is uncertain, although they have been incriminated in outbreaks of food poisoning. Bargen's coccus, described as occurring in chronic ulcerative colitis, is apparently a member of this group.

Pneumococci

Diplococcus Pneumoniae (*The Pneumococcus of Fraenkel*) (*Pasteur and Sternberg, 1881; Fraenkel, 1884*). The pneumococcus is by far the most common cause of lobar pneumonia (over 90 per cent) and may also cause bronchitis, bronchopneumonia, conjunctivitis and corneal ulcers, otitis media, brain abscess, meningitis, endocarditis, arthritis, peritonitis, and other conditions. It is frequently present in the normal mouth.

The following table (p. 2, Heffron's "Pneumonia," 1939) lists the organisms isolated from 3319 cases of lobar pneumonia.

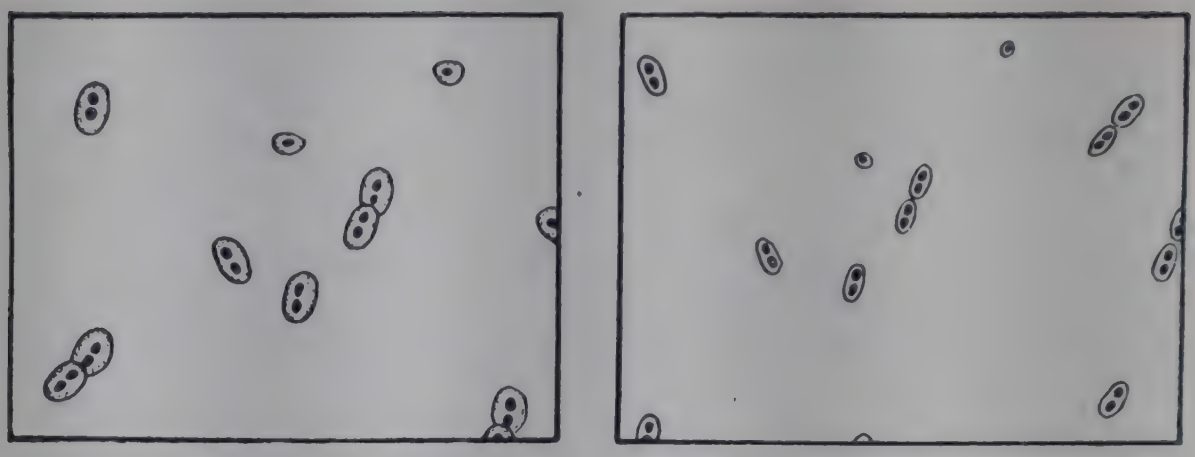
Table 3

Organisms Isolated in 3319 Cases of Lobar Pneumonia

	Pneumococcus	*Streptococcus*	*Friedländer's Bacillus*	*Influenza Bacillus*	*Staphylococcus*	*Mixed Infection*	*Total Cases*
Avery, Chickering, Cole, and Dochez (1917)	454	8	3	6	3	6	480
Cecil, Baldwin, and Larsen (1927)	1913	76	8	1	2	..	2000
Sutliff and Finland (1933)	822	10	6	..	1	..	839
Total	3189	94	17	7	6	6	3319
Per cent	96.1	2.8	0.5	0.2	0.2	0.2	100.0

Morphology. The pneumococcus in body fluids and in cultures in albuminous media appears as two lanceolate bodies with bases apposed (less frequently oval and rarely round) set in a capsule. In cultures on plain media it does not show a capsule. Short chains occur which are obviously made up of diplococci. Capsules are easily demonstrated by special stains. The organism is Gram-positive.

Cultural Characteristics. The pneumococcus does not grow at temperatures below 20° C. On plain agar it grows in very small dewdrop colonies which are slightly grayish by reflected light. It is best cultivated on blood or serum agar (pH 7.6 to 7.8). Deep colonies in blood agar show a zone of olive-green discoloration around each colony, whereas colonies on the surface look green by transmitted light, owing to alteration of the blood pigment. There is great variation in the smoothness of the surface of the



Pneumococci with capsules. Drawing, Naval Medical School.

colonies, depending upon the size of the capsules. Pneumococci which have large capsules produce smooth, often sticky colonies, whereas pneumococci with small capsules produce small, rough, granular colonies which may resemble closely those of the *Streptococcus viridans*. Some strains always show capsules; others are variable. Often the presence of a capsule is related to environment; i.e., capsules are more easily demonstrable in organisms taken from various body fluids, or from culture media containing serum or whole blood. Some colonies are flat and concentrically ringed, resembling a "checker man."

The pneumococcus ferments many common sugars, and also inulin, with the production of a considerable amount of acid. It grows readily in meat infusion broth with a pH of 7.6 to 7.8, causing a diffuse clouding of the medium. It grows profusely in media containing sugars, but is very quickly killed by the acid produced by their fermentation unless this is neutralized. Litmus milk is acidified and usually coagulated.

Viability. On ordinary media the pneumococcus loses its virulence and quickly dies unless transferred often. The best media for its preservation are highly buffered fluids such as blood, blood serum, or media containing these body tissues. In these virulence is well maintained, and if kept in the icebox the cultures usually remain viable for several weeks. To maintain strains in a smooth phase, and virulent, it is best to pass them through mice once a week. They may be kept alive and virulent for several years if dried and sealed under a vacuum.

Differentiation from Streptococcus. The pneumococcus can be distinguished from the *Streptococcus viridans* by its fermentation of inulin and especially by its solubility in bile (or 10 per cent solution of bile salts). It must also be distinguished from the pneumobacillus of Friedländer which, although it possesses a capsule like the pneumococcus, is a Gram-negative bacillus.

Types of Pneumococci. The differentiation of pneumococci into serologically distinct types by Neufeld, by Dochez and Gillespie, and by Cole and his colleagues, was of great practical importance. Originally Cole divided pneumococci into four groups. Groups I, II, and III were termed the fixed types: types 1, 2, and 3. Heterogeneous group IV included all pneumococci which did not belong to the three fixed types. Later work by Cooper and others resulted in the differentiation of group IV into many other types. On the basis of immunologic reactions the pneumococci have been divided into as many as 75 types. All are not of equal importance. During a two-year nation-wide study, including more than 22,000 type-determined cases of lobar pneumonia, 10 types (1, 2, 3, 4, 5, 6, 7, 8, 14, and 19) accounted for 74.6 per cent (Rumreich et al.). The following table shows the relative incidence of these 10 types according to data obtained by Finland (1942).

Since 1939 commercial diagnostic and therapeutic antiserum has been available for 32 types.

Table 4

RELATIVE INCIDENCE OF THE 10 MOST COMMON TYPES OF PNEUMOCOCCI

Pneumococcus Type	*Number of Cases*	*Per Cent of Total*
1	9,554	32.8
2	3,128	10.7
3	2,978	10.2
4	1,038	3.6
5	1,325	4.5
6	902	3.1
7	2,192	7.5
8	1,940	6.7
14	740	2.5
19	720	2.5
Total types 1–8, 14, and 19	24,517	84.1
Other types	4,655	15.9
Total number	29,172	100.0

Many normal individuals carry pneumococci in their throats. The types of these bacteria depend to a great extent upon the environment and contacts. Many of them are avirulent, although highly virulent strains may occur in normal throats also.

Specific Polysaccharides. Avery and Dochez and others have isolated from the capsular material of many types of pneumococci type-specific soluble substances which chemically are complex polysaccharides. The polysaccharide of each type differs chemically from that of every other type, and the type-specificity depends upon this chemical difference. Without these capsular substances all pneumococci are immunologically alike. Although the specific soluble substance is not directly toxic for animals, it endows the pneumococci with their virulence and invasive power. It neutralizes completely *in vitro* and *in vivo* the protective power of the homologous immune serum. It is found in the blood serum and body fluids during the active stage of the disease and disappears with recovery.

It is probable that the body defends itself against the pneumococcus by the production of an anticarbohydrate antibody. The outcome seems to depend primarily upon the relative quantity of specific soluble substance produced by the organism and of the antibody produced by the host. If sufficient antibody is produced, it neutralizes the specific soluble substance in the body fluids and in the capsules of the organisms. These bacteria are thus virtually degraded into forms which are phagocytable. It has been demonstrated by Dochez, Clough, and others that the serum of patients at crisis shows specific protective power, agglutinative and phagocytic activity for the infecting type.

Avery and Dubos (1930) extracted from a bacterium isolated from a peat bog an enzyme which exerted a specific lytic action on the capsular polysaccharide of type 3 pneumococcus. This enzyme destroyed their capsules and rendered the organisms avirulent and phagocytable. On injection it not only protected mice from virulent type 3 strains but exerted some curative action in experimental infection. This enzyme is neither bactericidal nor bacteriolytic but only deprives the type 3 pneumococci of their capsules; hence its therapeutic usefulness is very limited.

The immunizing properties of these specific polysaccharides have been investigated by several groups of workers, notably Felton, Francis and Tillett, and Heidelberger, McLeod and their collaborators. Experimental vaccination, by this last group, of over 17,000 volunteers in the Air Force Technical School gave results that show promise.

Virulence. Many types of pneumococci, when freshly isolated, are extremely virulent for laboratory animals, especially mice and rabbits. One millionth of 1 ml. of a broth culture when injected subcutaneously or intraperitoneally into a mouse is often sufficient to kill it in 24 to 48 hours. At autopsy the peritoneal cavity is teeming with organisms, and they can be recovered from the heart's blood. After prolonged cultivation on artificial media the virulence is much diminished but may often be restored by animal passage.

Variation. Griffith, Dawson, and others changed smooth pneumococci of the fixed type (S form) into rough pneumococci corresponding to the R form by growing the fixed types in their homologous immune serum or, less easily, by growing them under unfavorable cultural conditions. These so-called "degraded" strains had little or no capsular substance, were relatively avirulent, and did not react with the type-specific antisera. These investigators then changed these degraded strains into their original type or into other fixed types by injecting them subcutaneously into mice together with a fixed-type vaccine or a solution of an alcoholic precipitate of extracts of the specific soluble substance. The degraded forms then changed to the type of pneumococcus from which the vaccine was prepared; or, if not sufficiently degraded, reverted to their original type, with a corresponding increase in capsular substance and virulence. More recently Avery, MacLeod and McCarty (1944) have isolated from type 3 pneumococci an active substance apparently responsible for this transformation. When purified it was found to be a highly polymerized viscous form of desoxyribonucleic acid, with no demonstrable protein, carbohydrate, or lipid. Under appropriate conditions this fraction, in extremely minute amounts, has been shown to be capable of transforming unencapsulated R variants of type 2 pneumococci into fully encapsulated cells of type 3.

Laboratory Diagnosis and Type Determinations

Pneumococci are recovered from many sources. The materials most often examined are sputum, pleural exudate, blood, cerebrospinal fluid, and pus from middle-ear or mastoid infections. It is highly desirable, if possible, that the samples for study be collected before the patient has received either drug or serum therapy. If any of the sulfonamides have been given the addition of para-amino-benzoic acid to the culture medium (5 mg. per 100 ml.), as suggested by Janeway (1941), may result in growth of the pneumococcus.

It is well, first of all, to make a Gram-stained smear of the sample to determine the presence of the typical diplococci, their approximate number, and the presence or absence of contaminants. From the appearance of such a preparation the bacteriologist can usually determine whether or not the pneumococci may be typed directly by the Neufeld capsule-swelling technic, and whether isolation of the organisms should be accomplished by streaking the material on plates or by mouse inoculation.

Blood Cultures. Pneumococci are frequently found in the blood, although they may not be abundant. Routine blood cultures give valuable information in diagnosis and prognosis. It is important to obtain an adequate sample of blood and to dilute it sufficiently with the medium; i.e., 10 ml. blood in 100 ml. meat infusion broth. Addition of 0.1 per cent glucose encourages growth. Such cultures should be kept for several days as growth may be slow.

Isolation from Sputum. It is important to obtain the sample of sputum from the deeper air passages. With children and other patients who may be unable to raise a suitable sample from the lungs a pharyngeal swab may be used. Many mouth contaminants may be eliminated by washing the chosen particle of sputum in saline. The material may

then be streaked upon blood agar plates, which are examined after 18 to 24 hours of incubation for typical green-zoned pneumococcus colonies.

If the Gram-stained smear has shown very few typical cocci, or if contaminants are numerous, the washed sputum may be emulsified in saline, or ground up in a sterile mortar, and 0.5 ml. injected intraperitoneally into a white mouse. The mouse is so highly susceptible that the pneumococci usually rapidly outgrow other bacteria. Peritoneal fluid may be removed for examination by means of a capillary pipet; it will often contain enough pneumococci for typing within three to five hours.

If mice are not available Avery's "artificial mouse" may be used. This is a tube of beef or beef-heart infusion broth of pH 7.8, containing 0.3 per cent glucose and 5 per cent blood (preferably, although not necessarily, from a rabbit). It is inoculated with the emulsified sputum and incubated at 37° C. and examined frequently. As soon as the Gram-positive cocci become sufficiently abundant they are typed.

This broth culture and the mouse peritoneal fluid may be inoculated on blood agar plates if a pure culture is desired for future study.

Each of these three methods has its special advantages and disadvantages. Direct plating on blood agar reveals the other bacteria that may be present as well as the relative number of pneumococci. The mouse method suppresses these other bacteria and facilitates the growth of abundant pneumococci. The artificial mouse is a good medium for contaminants as well as for the pneumococcus.

Isolation from Other Materials. Pleural exudate in pneumonia, cerebrospinal fluid from meningitis, pus from otitis media and mastoiditis, or swabs from throat and conjunctiva are commonly streaked on blood agar plates and typical colonies studied.

Determination of Type. *Neufeld's capsule-swelling ("Quellung") reaction* is the method of choice, and has, for practical purposes, displaced other means of typing. Neufeld first observed that when the pneumococcus is treated with its homologous serum, the capsule of the organism becomes greatly swollen, the so-called "Quellung reaction." This swelling may affect also the capsular substance between the two elements of the diplococcus, so that they are more widely separated. This swelling does not occur with heterologous sera. This phenomenon has been utilized to determine the type of pneumococcus directly from the sputum. The technic may be used for materials from other sources and also for cultures. The procedure is essentially as follows: A small (about 1 mm. in diameter) loopful of sputum is placed on a clean coverslip. A large (4 to 5 mm. in diameter) loopful of the typing serum is added, and a small loopful of methylene blue; these are mixed thoroughly. The coverslip is inverted on a clean glass slide, and enough pressure used to make a thin, even film between the two layers of glass. This preparation is allowed to stand for a few minutes, preferably in an improvised moist chamber (i.e., a Petri dish containing a piece of moist filter or blotting paper), to prevent drying. It is then examined under the oil-immersion lens with the light partially cut down. A positive reaction is indicated by a clearly outlined unstained area, having a "ground-glass" appearance, surrounding the dark blue pair of cocci. The sharpness of outline of the capsule is of more importance than the degree of swelling, for some pneumococci have very narrow capsules. If no capsule swelling is observed within a few minutes the preparation, in a moist chamber, may be incubated for about 30 minutes. If no positive reaction develops some of the material should be inoculated into a mouse or into a tube of Avery's broth. If pneumococci are present typing can be done with the peritoneal exudate or culture.

If decisive results are not obtained by capsule swelling a pure culture of the suspected pneumococcus should be obtained and studied for bile solubility and inulin fermentation. For testing bile solubility, 0.1 ml. bile or a 10 per cent solution of bile salts is added to 0.4 ml. broth culture. The inoculated culture medium is incubated at 37° C. and observed every 15 minutes for an hour. Adequate controls should be included. A pure culture of pneumococcus should dissolve in the bile.

Although the capsule-swelling technic seems simple, it requires skill and experience for its accurate interpretation, and reliable materials are a necessity. The typing sera are prepared in rabbits. The reaction is a quantitative phenomenon and suitable proportions of reagents must be used. Too many pneumococci may result in agglutination or in absorption of the antibody by the specific soluble substance present. Sometimes several loopfuls of serum are needed. With practice, skill and judgment can be developed.

Obviously, testing each sample of sputum with 32 to 75 typing sera is inconvenient. Pools of sera ("A" to "F" inclusive) are available with which the material can first be examined. If a positive reaction is obtained in one pool, the sputum may then be tested with sera representing each type in the pool. Both typing and pooled sera can be obtained from manufacturers of biologic products. Minimum requirements have been established for these diagnostic sera (National Institute of Health, Washington, D.C.).

It is always possible that the organism found may be a pneumococcus of a type not represented in the sera. With some samples of sputum positive reactions will be obtained with sera of more than one type. Certain types are related to each other, and, in such cases, a crossing may be expected to occur. On the other hand, there may actually be two types of pneumococci in the specimen. In such a case a second sample should be examined, and an attempt made to find out, by blood culture and by mouse passage, which type is the etiologic agent.

Type Determination by Precipitation. This may be done with any material which contains sufficient specific soluble substance of the pneumococci; as, for example, urine, blood, cerebrospinal fluid, extracts of sputum, the supernatant fluid of centrifuged mouse peritoneal washings, or culture filtrates. Although such precipitin tests are not commonly done, such a technic may be very useful in samples in which pneumococci have become scarce or autolyzed, or when the material is greatly contaminated. To perform such a test the clear fluid, presumably containing the antigen, is carefully layered over the serum in a small precipitin tube. A "ring" of precipitate at the junction of the two fluids indicates a positive reaction.

Treatment of Pneumococcus Infections

Therapeutic sera are available for 32 types of pneumococci. There are official standard sera for comparative evaluation of those for types 1, 2, 5, 7, and 8, and minimum requirements have been established for those of types 3, 6, and 9 to 34 (omitting 26 and 30). Almost all of these antisera are now prepared in rabbits (Horsfall and Goodner, 1936) and are concentrated. These rabbit sera possess many advantages over the horse sera formerly used. They are usually given intravenously. In a classification involving so many types confusion is avoided by use of arabic numerals.

These specific sera have been very effective in lowering the mortality in pneumococcus pneumonia, but the results with sulfonamide therapy and with penicillin have been so outstanding that serum therapy is little used at present. Drug therapy is much more convenient and far less expensive. A combination of chemotherapy and serum therapy is sometimes advisable. It is important to know the type of pneumococcus and if a bacteremia is present. Specimens of both sputum and blood should be collected for such typing before the drug is given, if possible.

Chemotherapy. Chemotherapy is now a routine method of treatment. Many of the sulfonamides have been used successfully in pneumococcus infections, and especially in the pneumonias. Sulfadiazine and sulfamerazine are probably the drugs of choice at the present time.

Penicillin. This agent is proving most valuable in the treatment of pneumococcus infections. A special field of usefulness is found in infections involving various body cavities, since this drug can be injected directly into them and exert a local as well as a general action. In pneumococcus meningitis it is injected intrathecally; it can be given intraspinally, intracisternally, or even into the ventricles in case of spinal block. In

empyema the pus removed by drainage may be replaced by penicillin, and a thoracotomy often avoided. Penicillin may be given intraperitoneally in pneumococcus peritonitis. It may even be injected into the pericardium. A combination of sulfonamide and penicillin therapy is sometimes used.

Prophylactic Vaccines. These have been tried extensively, but the results have been inconclusive. MacLeod et al. (1945), however, in a carefully controlled experiment in a large military establishment, found that immunization with the capsular polysaccharides of four types of pneumococci largely eliminated pneumonia caused by these types among the vaccinated, although the incidence of pneumonia due to types not included in the vaccine was not affected.

Sarcina Forms

Sarcina forms of cocci are best observed in hanging-drop preparations, in which they can be seen as little cubes, like parcels tied with string. By noting them when they are turning over it will be seen that they are different from the tetrads which divide in only two dimensions of space. At times the packet formation is not perfect and it is difficult to distinguish such as sarcinae. All sarcinae are Gram-positive. If the staining of sarcinae be too deep it may obscure the lines of cleavage.

Various sarcinae have been isolated from the stomach, especially when there is stagnation of stomach contents. Sarcinae have also been found in the intestines. In plates *S. lutea* is frequently a contaminating organism, since it is often present in the air. The demonstration of sarcina morphology should always be made from liquid media.

Other Gram-positive Cocci

It is convenient to divide all cocci which do not show chain or packet formation into two classes; namely, those which are Gram-positive and those which are Gram-negative.

Gaffkya Tetragena (*M. tetragenus*) *Gaffky, 1881.* This organism is frequently found associated with other organisms in sputum, especially with tubercle and Pfeiffer bacilli. Smears from sputum or pus show large cocci arranged in groups of fours surrounded by a broad capsule. In cultures the capsule is often absent. The colonies, which are rather slow-growing, are white, slightly smaller than those of staphylococci and are quite viscid. *Gaffkya tetragena* does not liquefy gelatin but produces acid in glucose and lactose. Milk is slightly acidified, and is usually coagulated in one to three days but the coagulum is not digested. This microörganism may be responsible for abscesses about the mouth, especially the teeth. Injected subcutaneously into mice, it produces a fatal septicemia in three or four days and the blood shows great numbers of encapsulated tetrads. Its chief pathogenic role is that of a secondary invader, causing purulent inflammations. It has been reported as a cause of septicemia in man.

Staphylococci

The staphylococcus (Ogston, 1881) is a round, Gram-positive coccus which divides irregularly into masses which have been likened to clusters of grapes. Staphylococci have been grouped roughly into types according to their pigment production—*Staphylococcus albus, Staphylococcus aureus,* and *Staphylococcus citreus*—although this characteristic is a variable one. The Sixth Edition of Bergey's Manual has placed the old genus *Staphylococcus* in the genus *Micrococcus.* Those staphylococci of chief medical interest are found under the species *Micrococcus pyogenes,* var. *albus; M. pyogenes,* var. *aureus;* and *M. citreus.* Other micrococci may at times be pathogenic also. Regardless of the new generic name these familiar microörganisms will no doubt be commonly called "Staphylococci."

S. albus is commonly found on the skin, and in the nose and throat. It is occasionally pathogenic. A variety called *S. epidermidis* has been found in stitch abscesses. *S. citreus* produces a lemon-yellow pigment in culture, and is only feebly pathogenic. *S. aureus* produces a golden-yellow colony, and is the common cause of various suppurating conditions.

Cultural Characteristics. Staphylococci grow readily at room temperature but better at 37° C., on all the ordinary media. They acidify and coagulate milk, liquefy gelatin, and produce a uniform turbidity in broth. Glucose, lactose, and saccharose are fermented with the production of acid, but not gas. Coagulated serum media may be slightly liquefied. Pigment production is especially abundant on potato. On blood agar there is a variable amount of hemolysis around the colonies. Staphylococci are exceptionally resistant to desiccation, and cultures or dried pus may contain viable organisms for months. They are more resistant to heat than most vegetative forms of bacteria, and temperatures of 58° to 60° C., for one-half hour may be required in the preparation of heat-killed vaccines.

Virulent staphylococci produce coagulase; nonvirulent strains do not. The *coagulase test* is rapidly coming into routine use in laboratories as a quick and practicable method of determining the potential pathogenicity of a given strain. No other bacteria are known to give this reaction. Gillespie's (1943) modification of a method described by Fish (1940) is recommended as a simple technic for performance of the test. To 0.5 ml. of a 1 : 20 saline dilution of human plasma (oxalated or citrated) approximately 0.1 ml. of an 18- to 24-hour broth culture is added with a capillary pipet. After one and after three hours of incubation at 37° C. the culture is examined. It is then left at room temperature overnight and again examined. With pure cultures a coagulum often appears within one-half to one hour. An emulsified agar culture of suitable opacity may be used instead of broth. Positive and negative controls should be included, as well as a sterile broth control when indicated. The presence of glucose in the medium inhibits formation of a coagulum, as does the presence of proteus or hemolytic streptococcus organisms.

Poured plates of a clear agar medium containing 20 per cent human plasma may be used for the coagulase test (Reid and Jackson, 1945). Numerous strains may be tested on one plate if small (4-mm.) areas are heavily inoculated with growth from solid media. A positive reaction is indicated by a gray halo of variable diameter. Plasma from rabbits and from several other animals has been used.

Variation. Hoffstadt and Youmans have dissociated smooth *S. aureus* cultures into rough forms and have obtained the small G type of colony composed of minute filtrable forms. These dissociated forms differed from the original in the lack of pigment production, and of pathogenicity.

Toxins. *S. aureus* and even some strains of *S. albus* produce certain toxins in varying amounts. Filtrates of old broth cultures contain a ferment-like substance, leukocidin, which disintegrates leukocytes. Many of the virulent strains also elaborate a hemolysin which may be demonstrated *in vivo* or *in vitro*. A natural antileukocidin and antihemolysin may occur in normal blood and can be produced in animals by injection of the toxin. A tissue-necrotizing toxin has been found which produces a local lesion in the skin, akin to an abscess, on intradermal injection. Certain strains elaborate a lethal toxin which causes sudden death on subcutaneous or intravenous inoculation. Whether these activities are different manifestations of the same toxin or of distinct substances is unsettled.

Outbreaks of food poisoning due to a staphylococcus toxin from contaminated food are frequent. In staphylococcus food poisoning the incubation period is brief and the illness of short duration. The symptoms are nausea, vomiting, diarrhea, and prostration. If staphylococci are found in a suspected food, the toxin may be demonstrated by injecting filtrates of the culture intravenously (0.5 to 5.0 ml.) into healthy adult cats or intra-

peritoneally into kittens. Vomiting is the characteristic reaction produced by the enterotoxin. If the animal is given a meal before the injection the results will be more decisive. The enterotoxin is relatively heat-stable, and other lethal toxins in the culture filtrate may be eliminated by selective heating. The enterotoxin will not, however, withstand boiling.

Pathogenicity. *S. aureus* and occasionally virulent strains of *S. albus* are the common cause of boils, abscesses and carbuncles, and other skin infections. Infections of the tonsils, sinuses, middle ear, and mastoid are frequently caused by staphylococci. From such infections the organisms may get into the blood stream and cause a septicemia or pyemia with multiple secondary abscesses in the kidney, liver, and other organs. Endocarditis and suppurative lesions in the joints may occur. Osteomyelitis is most frequently caused by the staphylococcus, which is carried to the bone by the blood stream from a distant focus. Pyelitis and pyelonephritis may be due to this organism. Long-continued infection may result in amyloidosis. Staphylococci may be responsible for pneumonia and pulmonary suppuration, and are frequently found in empyema. Impetigo contagiosa and pemphigoid eruptions in children are often staphylococcal. In the tropics, where resistance is often lowered and skin infections are common, these infections often show great virulence, and long-continued fevers are often staphylococcal septicemias. Invasion of the blood stream by staphylococci occurs frequently as a terminal event or after death, so that these organisms are often found in postmortem blood cultures. A true septicemia due to *S. aureus* has a high fatality.

S. albus is a common contaminant of blood cultures, and when found must be regarded with great skepticism. However, it does (rarely) cause fatal septicemia.

Immunity. Injection of staphylococci into animals causes the formation of demonstrable antibodies including (with some strains) antitoxins. Attempts have been made to separate staphylococci into groups by means of agglutination tests. Such groups, however, are not well defined, and do not correspond in virulence or pigment production.

Julianelle and Wieghard (1934, 1935) isolated from staphylococci a complex antigenic protein common to all types, and two specific polysaccharides (haptenes), one (A) common to the pathogenic strains, and the other (B) present in the saprophytic strains. These can be identified by means of precipitin reactions with the serum of rabbits immunized by injections of the whole organisms. The pathogenic strains are coagulase positive.

Treatment of Staphylococcus Infections. Vaccines, toxin, and toxoid preparations have been useful in the treatment of some staphylococcal infections. Autogenous vaccines seem to be more effective than those prepared from stock cultures. Intracutaneous reactions to injections of toxin are used as a test of susceptibility and an indication for treatment with toxin or toxoid. Serum therapy has been useless. Recently, however, antitoxic sera which hold some promise have been obtained.

Bacteriophage therapy has been used in some staphylococcus skin infections and in cystitis due to staphylococcus infection. The bacteriophage is injected into the affected area and also applied locally in the form of wet dressings.

Such specific biologic therapy has now been largely supplanted by treatment with sulfonamides and antibiotics. Among the sulfonamides, sulfathiazole and sulfadiazine are more effective with the staphylococci than the others now in general use. Many staphylococci are resistant to these drugs; resistance acquired is apt to be permanent, and not associated with any diminution of virulence (Spink et al., 1945). Almost all staphylococci, both coagulase-positive and coagulase-negative, are susceptible to penicillin. Occasional strains have a natural resistance which is relative and may be overcome by larger doses. Resistance may also be acquired *in vivo* and *in vitro*. In infections due to strains resistant to crude penicillin it is well to consider the use of penicillin X, G, K, or F. In staphylococcus infections penicillin is administered as in the previously discussed pneumococcus infections; i.e., intravenously, into serous cavities, or locally. Its use in early osteomyelitis may make operation unnecessary; in chronic cases it becomes an adjuvant. In septicemias

supplementary treatment with sulfonamides and transfusions is helpful. Penicillin compresses may be used in skin infections.

An enzyme called *penicillinase* is produced by resistant staphylococci as well as by some spore-bearing and Gram-negative bacilli. It neutralizes the action of penicillin. This enzyme may be put to very practical laboratory use by adding it to the medium when materials from penicillin-treated patients are to be cultured.

Gram-negative Cocci—Neisseria Group

The organisms in this genus are Gram-negative diplococci which are characteristically flattened at their opposed surfaces so that they are shaped like a biscuit or coffee bean. Two species are of great importance, the gonococcus and the meningococcus. Other members of the group are occasionally involved in infections also, but they are important chiefly because they may be confused with these two pathogenic species.

Gonococcal Infections

Neisseria Gonorrhoeae (*Gonococcus*) (*Neisser, 1879*). The gonococcus is the cause of gonorrhea, gonorrheal ophthalmia, and occasionally of systemic manifestations such as gonorrheal arthritis, endocarditis, meningitis, and septicemia.

Morphology. The organism is typically plano-convex or biscuit-shaped, although in old cultures or in secretions from chronic cases there is a tendency to involution forms. In the secretions, gonococci are generally found grouped in masses of several pairs, most characteristically within the pus cells, or on epithelial cells, but they are also found extracellularly. They can usually be identified readily with a Gram stain.

Cultural Characteristics. The gonococcus grows only at temperatures of from 30° to 38° C., preferably 35° or 36° C., in enriched media which are sufficiently moist. It will not grow on plain media unless considerable pus is carried over in the inoculation. (For special media see p. 343.) On chocolate or blood agar the colonies appear as small, discrete, dewdrop spots, at first round, and later showing a slightly irregular margin. Cultures die out within 4 to 10 days unless subcultured on suitable media or covered with oil. The gonococcus produces acid in glucose but not in maltose, while the meningococcus produces acid in both.

Viability. The organism is killed in five hours or less by a temperature of 42° to 45° C. and speedily by ordinary drying. In moist smears of pus it may live for one or two days. When dried *in vacuo* from a frozen state, cultures of gonococci will live for many months.

Serologic Differentiation. Differentiation of gonococci has been attempted by different investigators by means of agglutination tests, and a number of ill-defined groups have been found.

Pathogenicity. Animals do not contract true gonorrhea, although local necrosis with some systemic reaction occurs on subcutaneous or intravenous inoculation, and immune sera can be produced.

Gonorrhea is the commonest of the venereal diseases. Its great importance from the public-health standpoint lies in the fact that an individual may remain infectious for years after the symptoms subside. In the male the gonococcus infects the urethra, and may invade the periurethral tissue, the prostate, seminal vesicles, and epididymis. In the female the favorite sites for the organisms are the urethra and the cervix uteri. From here the infection may spread to the tubes, ovaries, and pelvic peritoneum. It is one of the commonest causes of sterility in males as well as females. For diagnosis, separate smears should be made from the urethral meatus and from the cervical canal. The vagina of the adult does not provide a suitable soil for the development of gono-

cocci. In female children, however, the gonococcus produces a vulvovaginitis, which is extremely contagious, and in hospitals and institutions this infection may spread with great rapidity.

Laboratory Diagnosis. Diagnosis is made by demonstrating the gonococci in smears from the urethral or cervical discharge and by isolating and identifying the organism in cultures. In the acute stage, when the discharge is abundant, gonococci are found in large numbers, chiefly in the pus cells; later on, in the chronic cases, the pus cells largely disappear, and the organisms are found in smaller numbers on the epithelial cells. In men, secretion must be obtained by massage of the prostate and seminal vesicles.

Exercise, eating stimulating food, and if necessary the passage of a sound are useful as preliminary measures in obtaining prostatic secretion. The glans and meatus should be cleansed, and part of the urine is voided to wash out the urethra. The prostate is then massaged in the usual manner, and the secretion collected in a sterile dish. The rest of the urine is then voided into a sterile flask. Cultures and stained films are made from the prostatic secretion and the sediment from both specimens of urine. The prostatic secretion and urine sediment will usually contain also any organisms present in the urethra. In such smears gonococci are often atypical in their morphology with many distorted shapes and involution forms. In urinary sediments Gram-negative coccoid forms of colon bacilli may be present which may be intracellular, and thus may be mistaken for gonococci. It is, therefore, important to continue the search until some typical diplococci are found. There is nothing requiring greater discrimination than making a diagnosis from such a smear. It must be remembered that other members of the genus Neisseria, even including the meningococcus, may be found occasionally in urethral and vaginal smears.

Cultures. Diagnosis by cultures is more reliable than that by stained smear, and is the method of choice. The certainty of the diagnosis when made by culture makes this method much superior to smears, particularly in clinically questionable cases.

Materials for culture are most commonly collected with the usual cotton-tipped swabs. Carpenter (1945) recommends using two swabs for each sample: one for a "rolled-on" smear and the other for cultures. The specimens are usually taken from the urethra or cervix. In chronic cases in the male, prostatic secretions may be obtained; in vulvovaginitis in female children, vaginal secretions are examined. Sometimes material is taken from Bartholin's gland abscesses, from rectal secretions, and from the conjunctiva. Samples of blood, spinal fluid, and joint fluid are often examined; these fluids are collected with a suitable syringe and transported in tubes. The swabs for culture may be plunged into a small tube containing some soft chocolate agar (Hirschberg, 1945); these tubes may be mailed. Koch (1947) has shown a positive correlation between the cycle changes in the pH of the cervical mucus and the ability to isolate gonococcus from cervical cultures.

Plates are streaked in the usual manner. Chocolate agar, with a meat infusion base, is the medium preferred. Growth is encouraged by the presence of 2 to 18 per cent of carbon dioxide in the atmosphere. An adequate amount of carbon dioxide may be obtained by very simple means such as placing a lighted candle in a jar containing the inoculated plates and putting the lid on tightly. Necessary moisture may be supplied by placing a damp piece of filter or blotting paper in the bottom of the jar. Satisfactory growth of delicate colonies may occur in 24 hours, but 48 hours of incubation is often required.

The oxidase reaction is a useful aid in recognizing gonococcus colonies on mixed plates. A 1 per cent aqueous solution of para-amino-dimethylaniline monohydrochloride is dropped on the plate culture by means of a pipet, or placed on the suspected colonies with a medicine dropper. Those colonies which produce an oxidase first turn pink, then maroon, and finally black. All of the Neisseria, and some other bacteria as well, show

this change which usually occurs in about two minutes. The dye is toxic for the microorganisms, and the gonococcus is usually dead by the time the colony becomes black; so colonies should be fished while still pink if subcultures are to be made. The dye does not interfere with the making of Gram stains.

Further identification of the gonococcus may be made by fermentation reactions; the gonococcus produces acid in dextrose only. A table showing the fermentation reactions of the Neisseria is given on p. 55.

Serologic methods of identifying the gonococcus are not always satisfactory since "crossing" with other Neisseria, especially with group II meningococci, usually occurs. Agglutinating sera prepared in chickens (Phair, 1943) are said to give fairly specific results if used according to the technic of Noble (1927). See p. 54.

Gonorrheal Ophthalmia. One of the greatest advances in preventive medicine is represented by the Credé method of instilling silver nitrate into the eyes of the newborn, thus preventing ophthalmia and blindness in the offspring of gonococcus-infected mothers. In the adult the eye may occasionally become secondarily infected. The diagnosis is readily made by smears from the exudate.

Acute Gonococcal Arthritis. Acute gonococcal arthritis may occur in an individual infected with gonococci. It is often monoarticular, or, if polyarticular, one joint may be more severely affected than the others. In fluid from infected joints, which may be serofibrinous or purulent, gonococci may be demonstrated by smears and in culture. In the serofibrinous fluids the organisms are most likely to be found in the flecks of fibrin. Although infection of the joints is metastatic through the blood stream, positive blood cultures are not commonly obtained. In a small number of cases an acute endocarditis similar to that caused by the streptococcus develops.

Gonococcus Meningitis. This condition is more common than is generally realized. Patients usually give a history of previous gonococcal infection, but cases of apparently primary meningitis and also of uncomplicated septicemia due to the gonococcus have been reported (Branham et al., 1938).

Complement-fixation Test. This test is of value particularly in the differentiation of gonorrheal arthritis from that due to other causes. In preparing the antigen, several strains of gonococci are often used since serologic differences have been demonstrated. Cross fixation may occur with meningococcus infections.

Treatment of Gonococcal Infections. Local as well as systemic treatment should be carried out until at least three consecutive smears are negative for gonococci. The sulfonamides exert a marked curative action on gonorrheal infections. The urethral discharge is rapidly diminished, and infection of the posterior urethra and prostate can usually be prevented by the administration of one of these drugs. The most striking results are obtained by penicillin therapy. This is now the treatment of choice.

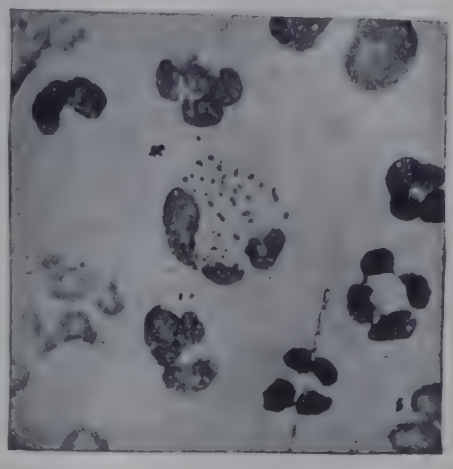
Epidemic Cerebrospinal Meningitis and Meningococcemia

Neisseria Meningitidis (*N. intracellularis, Meningococcus*) (*Weichselbaum, 1887*). This is the cause of a high percentage of the sporadic cases of acute cerebrospinal meningitis, and of almost all of the epidemic cases. The organisms are present in the cerebrospinal fluid and are usually demonstrable in the blood early in the disease.

The meningococcus may cause sepsis characterized by high fever, purpura, and

often hemorrhages into the adrenals with profound shock, without signs of meningitis (Waterhouse-Friderichsen syndrome). Death may occur within 12 to 24 hours in these fulminating cases. Early recognition and prompt treatment are essential.

Chronic meningococcemia, without meningeal symptoms, due to meningococci of relatively low virulence, may occur, especially in nonepidemic times. Diagnosis is often delayed in such cases, since the fever and rash suggest a number of other conditions. A positive blood culture is usually obtained readily from such patients.



Meningococci in spinal fluid. (Courtesy, Zinsser and Bayne-Jones: Textbook of Bacteriology, New York, D. Appleton-Century Co., Inc.)

Morphology. The meningococcus is a small, Gram-negative, biscuit-shaped diplococcus, and it is found both within and outside of the leukocytes in the cerebrospinal fluid. There is a greater tendency to variation in size and shape than is the case with the gonococci, which, in fresh material, are more uniform.

Cultural Characteristics. The meningococcus usually requires enriched media for growth from body fluids, although a semisolid agar with an infusion base is excellent for isolation. On blood agar, smooth, moist, slightly translucent colonies appear after 24 to 48 hours. They are usually about 1 or 2 mm. in diameter although they may be much larger. Later the center may become somewhat opaque and raised. The meningococcus grows best at 37° C. although growth may be obtained when the temperature is as low as 25° C. Temperatures higher than 37° C. are detrimental to meningococcus cultures. Meningococci ferment glucose and maltose, with the production of acid, but not lactose, saccharose, or levulose.

Viability. The meningococcus is sensitive to light and drying and its temperature tolerance, as has just been noted, has a narrow range. Material for culturing should be kept as near 37° C. as feasible, bearing in mind that meningococci will withstand lower temperatures better than higher. Newly isolated cultures may die out quickly, thus daily transfer is advisable for the first few days. Cultures will live for a month in a meat infusion semisolid agar and six months or more on tryptic digest agar containing yeast extract. The whole cultures may be kept at least two years when stored at —15° C. (Pabst, 1935). When dried *in vacuo* from a frozen state, and sealed *in vacuo*, they may be kept for several years at ordinary icebox temperatures.

Variation. As a rule meningococci that are newly isolated from spinal fluid or blood are "smooth," possessing specific capsular substance, and are virulent. Frequently those found in the nasopharynx of healthy carriers are "rough," avirulent, and have lost their specific capsular substance to the extent that they cannot be typed by agglutination. Many degrees of roughness and smoothness may be found, and all strains tend to become rough during long laboratory maintenance unless special precautions are taken. Rake (1933) has found both rough and smooth colonies in cultures from the spinal fluid and has produced rough strains in cultures from smooth strains.

Pathogenicity. The meningococcus is only feebly pathogenic for laboratory animals. If the culture is suspended in a solution of mucin and injected intraperitoneally, the virulence for mice is greatly increased, so that as few as two to ten organisms of some strains may be fatal (Miller et al., 1936). Mice so infected may be protected by specific

FOOD TECHNOLOGICAL RESEARCH

antiserum, and afford a satisfactory means of standardizing the serum (Branham and Pittman, 1940).

Classification. Dopter (1909) first noted serologic differences among meningococci and described two groups, the meningococcus and the parameningococcus. During World War I, French workers (Nicolle et al., 1918) reported four types: A, B, C, and D, most cases being due to A and B. Gordon and Murray (1915), by use of agglutinin absorption, separated their strains into four types: types I and III were related and corresponded with the French A; types II and IV were related and corresponded with the French B. There were no strains corresponding to the French C and D, which were said to be very rare.

During the 30 years since that time many hundreds of strains have been studied. Types I and III have been found to be indistinguishable epidemiologically, clinically, chemically, and immunologically. Only by absorption of agglutinins can a serologic difference be demonstrated. They are now placed together in group I (Rake, 1934, Branham, 1932). This group is responsible for over 90 per cent of epidemic cases (Branham, 1937), whereas group II, which is very heterogeneous, is found chiefly in sporadic cases and in chronic carriers. Recently a new group has been recognized. First reported by Cohen (1940), it was tentatively designated as group II *alpha* (Branham, 1942) for convenience although it is not a part of group II. Group II *alpha* is found in both epidemic cases and in carriers. Group IV strains have been too rare for their relationships to be determined. The classification now in common use, therefore, represents four groups (broader than types): I, II, II *alpha,* and IV.

Agglutinating sera for group determinations can be prepared by the intravenous inoculation of rabbits with increasing doses of dead or living cultures of the different groups. Excellent "typing" sera for group determination may be prepared by intravenous injection of chickens (Phair et al., 1943). Since these animals are insusceptible to the meningococcus or its products, very large doses may be given. This is a special advantage with group II strains which are poor antigens. If chicken sera are used, it is necessary to employ the rapid method (Noble, 1927) for agglutination (see p. 54); otherwise marked "crossing" is apt to occur.

Diagnosis. It is important that material for diagnosis be taken from the patient, if possible, before specific treatment has been begun. After sulfonamide drugs have been administered it may be difficult or even impossible to recover the microörganisms. Janeway (1941) has shown that the effect of the sulfonamides may be neutralized to a great extent by the addition of para-amino-benzoic acid to the culture medium to give a final concentration of 5 mg. %. But even with this method a sample of blood or spinal fluid from an untreated patient gives most constant results. Blood cultures should always be made as soon as possible and repeated if there are signs of sepsis. About 5 to 10 ml. blood, drawn into a sterile syringe from a vein, is added to 100 ml. of warmed infusion broth in a 250-ml. flask. Addition of 0.1 to 0.2 per cent dextrose may enhance growth. As soon as the first signs of meningitis appear, a lumbar puncture should be undertaken, and stained smears and cultures from the fluid should be made immediately.

A smear of the centrifuged sediment from the spinal fluid, stained by Gram's method, is an important factor in early diagnosis. The flattened Gram-negative cocci may be abundant both within and outside of the leukocytes; or careful search may be required for their detection; sometimes they cannot be found. A second smear may be stained with methylene blue, as this dye is especially valuable in

revealing morphologic characteristics. An additional film may be prepared and stained by Wright's or a Giemsa method for a differential count. This is a special aid to diagnosis when the fluid is only slightly cloudy, as a predominance of polymorphonuclear leukocytes will rule out the possibility of some other infections.

Pneumococci or streptococci in the spinal fluid are easily differentiated from meningococci by the Gram stain, as well as by culture. The influenza bacillus can be distinguished by its structure; filamentous forms are common in the spinal fluid. Tuberculous meningitis may produce a cloudy fluid, but the cells are chiefly lymphocytes. Acid-fast stains should be made on such fluids. Exceptionally, fluids from patients with encephalitis and poliomyelitis may be cloudy, and no organisms are demonstrable by smears or by culture. However, frank purulence in a fluid which contains no organisms is usually due to a meningococcus meningitis and immediate treatment for this disease should be instituted.

For cultures a generous inoculum should be used (i.e., from 0.5 to 1.0 ml., depending on the nature of the fluid) on a blood or chocolate agar plate and into a tube of semisolid agar medium, with or without enrichment. Centrifuged sediment may be used, or uncentrifuged spinal fluid when the meningococci seem to be especially abundant in the stained smear. The spinal fluid itself as a medium should not be ignored. Incubating it at 35° to 37° C. overnight often results in a multiplication of the meningococci so that their detection in smears and their cultivation are easier. Plate cultures should be kept moist while incubating. Some workers use a candle jar, as for the gonococcus, for this purpose.

Meningococci are present in the petechiae which occur in meningococcic infections. They may be demonstrated by withdrawing material by means of a capillary pipet or fine hypodermic needle, preparing stained smears, and making cultures of the organism in semisolid medium. In fatal fulminating cases demonstration of meningococci in the purpuric skin lesions may allow a diagnosis when other methods are unsuccessful.

Colonies of meningococci on blood agar plates are usually characteristic in appearance, and may be confluent. They may be fished to semisolid medium or to blood agar slants for further study. On semisolid agar the meningococci grow as a pellicle at the surface of the medium.

For fermentation tests, growth from a pure culture is inoculated into a special semisolid agar medium containing the desired carbohydrate and an indicator. The meningococcus produces acid from dextrose and maltose. Individual strains vary in their ability to ferment these sugars, and sometimes the reaction is very transient. Fermentation becomes more typical after a period of laboratory maintenance.

For clinical purposes the demonstration of a Gram-negative intracellular diplococcus in the spinal fluid is presumptive evidence that the case is one of meningococcus meningitis. For further identification of the organism, however, it is necessary to determine its growth on culture media, including its fermentative abilities, and also its agglutinability in a polyvalent immune serum prepared from strains of the main serologic groups. Determination of the group by use of monovalent sera should be made also.

DIAGNOSIS BY CAPSULE SWELLING. Meningococci of groups I and II *alpha* will show capsule swelling with appropriate sera if the strains are "smooth." No capsules have been demonstrated on group II strains. Thus the Quellung reaction of Neufeld may be used for rapid identification and determination of serologic group. If the meningococci are sufficiently abundant to be easily seen and the sample has been taken before sulfonamide therapy has been instituted, a direct "typing" may be made with the spinal fluid within

a few minutes. Or this method may be used later with a pure culture. The application of this technic to the diagnosis of meningococcus infections was first made by Clapp (1935) and has proved valuable and time-saving. The technic is identical with that for the pneumococcus. Reliable sera must be used.

MACROSCOPIC AGGLUTINATION. In following the regular method, a series of dilutions of an immune polyvalent serum from 1 : 10 to 1 : 400 or higher is prepared according to the titer of the serum. Into a series of small tubes is placed 0.5 ml. of each dilution. To each tube is added an equal volume of a homogeneous suspension of the organisms. The suspension is prepared by washing off a 5- to 18-hour blood or serum agar culture with enough salt solution to give a definite but rather faint turbidity (i.e., approximately 1,000-000,000 meningococci per ml.). A control tube containing normal horse serum (or salt solution), instead of the immune serum, is essential. The tubes are incubated for two hours in a water bath at 37° C., and stored in an icebox overnight before being examined. The organism is identified as a meningococcus if clumping occurs in the higher dilutions of the immune serum and not in the control tube.

To determine the group to which the meningococcus belongs, another series of tubes is set up containing similar dilutions of each monovalent serum.

Noble's (1927) rapid method of agglutination gives excellent results and is preferred by many for its specificity and speed. The suspension of microörganisms contains five times as many bacteria, and the serum concentrations are five times as great as for the regular method. Only 0.1 ml. of serum dilution is placed in a tube, and 0.1 ml. of suspension added. The rack is rocked back and forth through an angle of about 90 degrees for two minutes in such a way that the mixture flows up the tube about 1 inch. Then 0.8 ml. of 0.85 per cent saline is added to each tube and the agglutination read at once. The usual control tubes are included. This rapid method must be used if chicken serum is employed, otherwise "crossing" will cause confusion.

NASOPHARYNGEAL CULTURES. Cultures from the nasopharynx are used chiefly for the detection of carriers. A bent wire, or straight wooden, applicator with a sterile cotton tip is introduced behind the soft palate and passed over the posterior nasopharyngeal wall. The material obtained is immediately spread over a blood agar plate which should be warmed previously to body temperature. Suspicious colonies are subcultured the following day, examined microscopically, and further identified. Other Gram-negative diplococci (*N. catarrhalis, N. flava,* and *N. sicca*) may be present in the normal pharynx and must be differentiated by their cultural characteristics and by their agglutinability in normal horse serum. From these subcultures macroscopic agglutination tests with the polyvalent serum are made as has been described.

TREATMENT OF MENINGOCOCCUS INFECTIONS. The sulfonamides have proved highly successful in the treatment of meningococcus infections. Although the meningococci are, as a rule, susceptible to nearly all members of this group of drugs, sulfadiazine and sulfamerazine are the most widely used at the present time. Usually these drugs are administered orally at intervals necessary to maintain the desired blood level. To very ill patients the initial doses are often given intravenously in soluble form. In the majority of epidemic cases, sulfonamide therapy proves adequate. Sometimes cases are encountered which fail to respond to this group of drugs, and other supplementary agents are indicated. Such cases are most often found among the very old and the very young. Also there are patients in whom intensive sulfonamide therapy is contraindicated. Individual strains of meningococci vary greatly in their susceptibility to sulfonamides; insusceptible strains are most apt to be encountered in sporadic cases.

Penicillin is especially useful in cases that do not respond well to sulfonamides. This antibiotic agent is less convenient to use than are the "sulfa drugs" as it is necessary to give it intrathecally, as well as intravenously, in cases of meningitis. The combination of penicillin with one of the sulfonamides is highly successful. A few penicillin-resistant strains of meningococci have been reported.

Monovalent, group-specific serum, prepared in rabbits and concentrated and refined, can be a valuable adjunct in treating the patients who do not respond well to drug therapy. The possibility that serum therapy may be needed constitutes a strong argument in support of routine typing of early cultures since cross protection between groups is lacking. Such serum is usually given intravenously; rarely, intrathecally. The old-fashioned polyvalent horse serum, which was the only specific treatment for cerebrospinal fever for many years, is no longer indicated.

Recently several cases of the dread Waterhouse-Friderichsen syndrome have been successfully treated with adrenal cortical extract, transfusions of whole blood or plasma, and sulfonamides.

Neisseria Catarrhalis (*Micrococcus catarrhalis*) (*Seifert, 1890*). This organism resembles the meningococcus but can be differentiated by agglutination and cultural procedures. It grows on plain agar and at room temperature.

Original cultures may show only slight growth, whereas subcultures prove luxuriant. The colonies are larger, more opaque, and have a more irregular wavy border than the round colonies of the meningococcus. The colony emulsifies readily, but the microorganisms have a tendency to settle. It does not ferment any of the sugars. *N. catarrhalis* occurs in the normal nasopharynx and is generally considered nonpathogenic. However, it is occasionally responsible for sporadic cases of meningitis, and has been reported in various respiratory infections, conjunctivitis, and occasionally in septicemia and endocarditis.

Pigmented Neisseria. Four species are recognized: *Neisseria perflava, N. flava, N. subflava,* and *N. flavescens.* The first three are found in the normal nasopharynx; *N. perflava* is usually most common. These have typical Neisserian morphology and the colonies, when young, may be easily confused with those of the meningococcus. They are less exacting in their growth requirements than the meningococcus and develop a greenish to golden-yellow pigment by the second day. All have a tendency to spontaneous clumping in suspension. They are differentiated from each other only by fermentation reactions and Wilson (1928) has suggested that they may be actually varieties of one species. All three have occasionally been found responsible for sporadic cases of meningitis.

N. flavescens was found by Branham (1930) in an epidemic of cerebrospinal meningitis in Chicago. It was isolated from the spinal fluid of 14 patients, four of whom died. The growth requirements of this organism were as exacting as those of the meningococcus, but like the other pigmented species, it was longer lived. The colonies become a golden yellow, and the cultures ferment no sugars. *N. flavescens* comprises, by agglutination, an independent and homogeneous serologic group. Otherwise this organism

Table 5

FERMENTATION REACTIONS OF THE GRAM-NEGATIVE COCCI

	Glucose	*Maltose*	*Saccharose*	*Levulose*
N. meningitidis (Meningococcus)	+	+	—	—
N. gonorrhoeae	+	—	—	—
N. perflava	+	+	+	+
N. flava	+	+	—	+
N. subflava	+	+	—	—
N. flavescens	—	—	—	—
N. catarrhalis	—	—	—	—
N. sicca	+	+	+	+

resembles the other three pigmented forms mentioned above. Its chief significance is that it seems to have been responsible for an epidemic of serious proportions, whereas the other forms have been found only in occasional sporadic cases.

Neisseria Sicca (*Micrococcus pharyngis siccus*). This small coccus forms white colonies, which are firm, stick to the medium, and do not emulsify easily. Growth takes place at room temperature. It is frequently found in the normal nasopharynx.

Table 5 gives the fermentation reactions of the various Gram-negative cocci.

CHAPTER 3

Spore-bearing Bacilli

Key and Notes

I. Genus: *Bacillus*. Grow aerobically. Mostly saprophytes. Often occur in long chains and form rhizoid colonies. Usually Gram-positive. Form of rod usually not greatly changed at sporulation.

A. Pathogenic forms.

1. *Bacillus anthracis*. Nonmotile rods with square-cut to concave ends, occurring in chains. Central spores.

B. Nonpathogenic forms. Usually motile, having central or eccentric spores.

2. *Bacillus cereus*. Very similar to *B. anthracis*. Usually nonpathogenic.

3. *Bacillus subtilis* group. Seventy-five or more species have been described. Some show pigment. Commonly found in soil, water, milk, intestinal contents. Occasional strains pathogenic. All grow well at room temperature.

II. Genus: *Clostridium*. Grow only anaerobically with a rare exception. Often parasitic. Many elaborate exotoxins.

A. Spores central, eccentric, or subterminal.

1. Rods swollen at sporulation.

a. Motile.

(1). *Clostridium fallax*.

(2). *Clostridium septicum* (*Vibrion septique*).

(3). *Clostridium chauvoeii*.

(4). *Clostridium novyii* (*B. oedematiens*).

(5). *Clostridium botulinum* (*B. botulinus*).

(6). *Clostridium sporogenes*.

(7). *Clostridium histolyticum* (*B. histolyticus*).

2. Rods not swollen at sporulation.

a. Motile.

(8). *Clostridium bifermentans* (*B. sordelli*).

b. Nonmotile.

(9). *Clostridium perfringens* (*B. welchii*).

B. Spores terminal.

1. Spores round.

a. Motile.

(10). *Clostridium tetani* (*B. tetani*).

2. Spores oval or elongated.

a. Motile.

(11). *Clostridium tertium*.

Spore-bearing Aerobes

Anthrax

Bacillus Anthracis (*Discovered by Pollander, 1849; Its Nature Recognized by Davaine, 1863, and Proved by Koch, 1876*). This is the only aerobic spore-bearing



Bacillus anthracis (in tissue exudate). (From Kelser and Schoening: Manual of Veterinary Bacteriology, Courtesy, Waverly Press, Inc.)

bacillus of medical importance. Anthrax was the first of all infectious diseases shown to be caused by a specific microörganism.

Morphology and Cultural Characteristics. This organism is a Gram-positive bacillus 5 to 8μ × 1 to 1.5μ. In cultures it has square-cut or concave ends and is often found in long chains, whereas in the blood of an infected animal it is in quite short chains, and the ends may be slightly swollen. It is nonmotile. A capsule may be demonstrated in preparations from the animal body. The spores are oval and centrally placed, and stain with difficulty.

B. anthracis grows well on all media and rapidly liquefies gelatin. On agar plates, virulent strains develop rough, "frosted-glass" appearing colonies, with wavy margins. Examination of the colonies under the low-power objective will show them composed of parallel wavy filaments, becoming less compact as they spread toward the margin. This structure is responsible for the term "Medusa-head" which is applied to these colonies. Milk is slowly acidified and coagulated. Spores develop most readily on potato media at a temperature of 30° C., and do not form at temperatures above 43° C. Spores are formed only in the presence of oxygen, and, therefore, are not found in the blood or tissues of infected animals. Anthrax spores are extremely resistant to physical and chemical means of sterilization.

B. anthracis has a mucin-like capsule composed of a polypeptide containing chiefly *d*-glutamic acid. This capsule is especially well developed in virulent strains, particularly in the body fluids of infected animals.

Variation. Variations in capsule formation and virulence occur. When the capsule is absent or small, the colonies are smaller and more mucoid than those of the encapsulated, rough forms. The smooth forms are less virulent. The attenuation of the organisms for vaccine by growth at high temperature causes a change from rough to smooth form.

Anthrax in Animals. Primarily a disease of the herbivora, anthrax is an important disease in domesticated animals, especially sheep and cattle. The characteristic pathologic lesion produced in animals implicates the spleen which becomes greatly enlarged, friable, and mushy. The mortality is from 75 to 100 per cent. Like cattle, guinea pigs, brown rats, mice, and rabbits are quite susceptible. Man is much less susceptible, but more so than the goat, horse, and pig. The white rat and the Algerian sheep have a high degree of immunity. Fowls are practically immune.

Spontaneous anthrax in animals is primarily intestinal, but soon becomes a generalized infection, and the blood is usually laden with the microörganisms before death. Although spores do not form in an intact body, they form in the presence of oxygen after a postmortem or the disintegration of the body brought about by maggots. For this reason the diagnosis should be made, without opening up the body unless it can be burned immediately, by staining the organisms in films of blood from the ear vessels. Dried spores live for years and can withstand boiling for hours.

Immunization of Animals. The mechanism of the immunity produced in animals is not known. The injection of dead organisms seems to have no effect. In Pasteur's method, which has given excellent results, two vaccines are cultivated at



(*Left*) *Bacillus anthracis* showing capsules. (*Right*) Colony of *Bacillus anthracis* on agar. (From Kelser and Schoening: Manual of Veterinary Bacteriology, Courtesy, Waverly Press, Inc.)

42.5° C. The first is attenuated for 15 days, and the second for only 10 days, and is given 12 days after the first. This immunity lasts for about a year. At this temperature only vegetative forms develop. Prolonged incubation destroys virulence for rabbits and then for guinea pigs and eventually for white mice. Several more convenient modifications of this original method of immunization have been developed.

Anthrax in Man. Man is chiefly infected by working with hides, hair, wool, or meat of diseased animals. Many infections have been traced to shaving brushes. The organism has been cultured from them, especially from new brushes. Brushes may be sterilized by soaking for four hours in a 10 per cent formalin solution at 110° C. Autoclaving at 15 pounds for three hours may be used for hair and bristles. Live steam at 100° C. kills the organisms in five to ten minutes.

The two most important types of anthrax infection in man are those due to handling products of diseased animals. They are: (1) malignant pustule, and (2) woolsorters' disease. An intestinal type, with a high mortality, from ingestion of infected meat eaten raw, is also known.

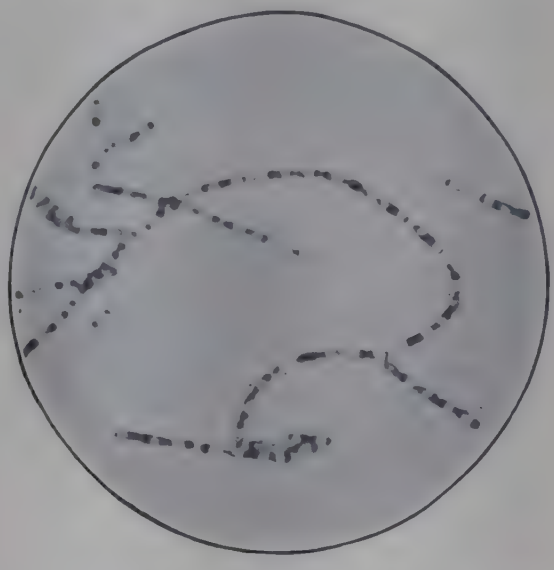
malignant pustule. This is due to inoculation of a cut or abrasion and, therefore, frequently occurs on the arms and backs of men unloading hides. It first appears as a pimple, and the center begins to show a vesicle, which changes into a black, necrotic area with a red, edematous areola. If the pustule is excised early, the prognosis is less grave. In taking material from a malignant pustule before excision, care must be taken to avoid rough manipulation, else bacteria may be expressed into the circulation. In fatal cases in which the pustule is not excised, a postmortem does not show the enlargement of the spleen and the abundance of bacteria in the kidneys seen in animals. Death seems to be due to toxemia rather than septicemia. A few cases with positive blood cultures have been reported. Cases have been reported in which recovery took place following intravenous injections of anti-anthrax serum.

woolsorters' disease. This type is a severe pneumonia with a high mortality, characterized by an edema of the lungs which is hemorrhagic about the bronchi, and by great swelling and edema of the bronchial and mediastinal glands. It is believed to be due to inhalation of spores.

Laboratory Diagnosis. The material to be examined is usually pus or fluid from a malignant pustule, blood in septicemia, sputum in the pulmonary form, and, rarely, spinal fluid from an anthrax meningitis. In the intestinal form, feces may also be studied. A Gram-stained preparation should be first examined for the large Gram-positive rods. It must be remembered that in preparations directly from the tissues there will be neither spores nor chains, but that capsules may be conspicuous. Agar plates and broth cultures are made and incubated at 37° C. Typical colonies are fished from the agar and further cultural identification made. A hanging drop is prepared from the broth culture to determine the lack of motility.

A very important procedure is the inoculation of mice or guinea pigs subcutaneously with the infected material or with the culture. The animals usually die in three to four days or earlier, and the organisms may be recovered from the

heart blood, liver, and spleen. An edematous gelatinous exudate may be seen at the site of inoculation. A very few of the bacteria may cause the death of the animal. A diagnosis of anthrax is made if the findings reveal a Gram-positive, nonmotile, spore-bearing, square-ended bacillus, producing characteristic colonies on agar plates and a fatal septicemia in animals.



Anthrax bacilli growing in a chain and exhibiting spores. (Kolle and Wassermann.)

For the detection of anthrax bacilli in shaving-brush bristles or in wool, the material is shaken vigorously with sterile salt solution. The solution is then decanted and centrifuged. The sediment is resuspended in 1 ml. of salt solution and heated to 80° C. for a half hour to kill all non-spore-bearing organisms. This may then be cultured, and injected subcutaneously into an animal.

DETECTION IN ANIMAL TISSUES: *Precipitin Test.* For the diagnosis of anthrax in decomposed tissues, Ascoli has devised a precipitin test. A piece of tissue is boiled in a few ml. of salt solution, which is then filtered until clear, and layered on the immune serum. In a positive reaction a precipitate occurs at the junction of the fluids. Suitable controls must be made, and a serum with known precipitating power must be used. The usual serologic tests (agglutination or complement fixation) are of no value in the diagnosis of anthrax infections.

Demonstration of capsules in body fluids from decomposed animals is valuable in differentiating anthrax from other contaminating organisms.

SERUM TREATMENT. In cases in which the infection becomes septicemic instead of localized, the outcome is usually fatal. In such cases one should inject anti-anthrax serum intravenously, in doses of 50 to 75 ml. In malignant pustule it is advisable to inject the serum in the subcutaneous tissues surrounding the lesion. The anthrax antiserum is made by simultaneous inoculation of animals with cultures of *B. anthracis* and antiserum. The best animal to use is the sheep and a preliminary vaccination by Pasteur's method is to be carried out.

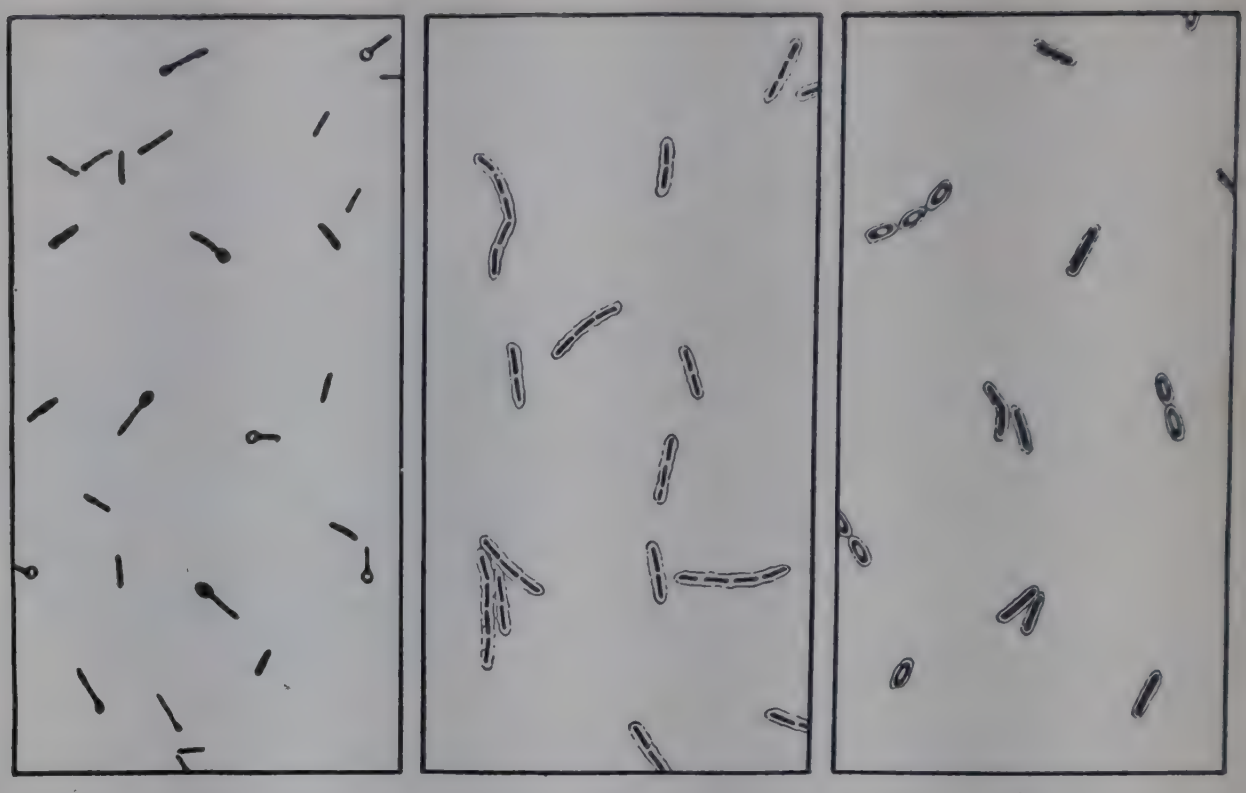
Recently arsphenamine, sulfathiazole, and penicillin have been used with some success.

BACILLUS CEREUS

Bacillus cereus is closely related to *B. anthracis.* It is a common inhabitant of soil. Although usually considered nonpathogenic, it has been known to cause localized lesions and has been isolated from spinal fluid and blood. An intensive study on the differentiation of *B. anthracis* and *B. cereus* has been made by Burdon (1947). *B. cereus* is less well known to bacteriologists than it should be. It is frequently confused with *B. subtilis.*

BACILLUS SUBTILIS

The name *Bacillus subtilis* (the "hay bacillus") is often loosely applied to a group of species of Gram-positive, aerobic, spore-forming rods. They are widely distributed in



(A) *Clostridium tetani.* (B) *Bacillus anthracis.* (C) *Clostridium perfringens* (*B. welchii*). (Drawing, Naval Medical School.)

nature, in hay, straw, dust, soil, milk, water, air, and practically everywhere. Thus they are frequent laboratory contaminants and may be secondary invaders in chronic suppurative lesions. They are usually nonpathogenic saprophytes.

The true *B. subtilis* is the type species of the genus *Bacillus*. This microörganism is motile, does not show a striking capsule, and may be differentiated from *B. anthracis* morphologically, culturally, biochemically, and by animal inoculation. A number of similar species have been described. A valuable study of this group of Gram-positive, aerobic, spore-bearing bacilli has been reported by Smith, Gordon, and Clark (1946). A key for their differentiation has been prepared by Burdon (1947).

Spore-bearing Anaerobic Bacilli

The anaerobic bacilli which occurred with great frequency in wounds during World War I are common inhabitants of the intestinal tracts of man and other animals, and hence are frequently present in fertilized soils. The spores of most of these anaerobes develop in the intestine, and may remain viable for years in the soil. The heavily manured lands of European countries are full of such spores, and cultures from the clothing of men in the trenches almost always showed anaerobic organisms, especially the Welch bacillus and frequently tetanus bacilli. The nature of the fighting in World War II was such that infection with these anaerobes was less common, but some instances of such infection did occur.

Isolation of these organisms in pure culture is technically exacting and much of the early work (prior to 1916) is untrustworthy, since many of those investigating these organisms were dealing with mixed cultures. Repeated plating out of cultures is usually necessary, and the selection of a single spore by the Barber technic has been found especially valuable in obtaining a pure culture.*

*Methods for obtaining anaerobiosis are described on p. 918.

Anaerobic bacilli do not thrive in living tissue. In order to produce infection there must be laceration with necrotic tissue or blood clot. War wounds contaminated with dirt and fragments of clothing, especially those produced by shell fragments, provide a suitable environment for their growth. Spreading occurs through injury to the surrounding tissues, both by the bacterial toxins and by mechanical pressure due to gas formation and edema. Proteolytic organisms digest the dead tissue, producing blackening and the foul odor of gangrene, although they do not form toxins and cannot invade healthy tissue.

The important organisms found in this gas gangrene of war wounds are divided into two broad groups according to their ability to ferment carbohydrates or digest proteins. This distinction is not clear-cut, since some of the saccharolytic organisms have a slight proteolytic action as well, and vice versa.

The more important saccharolytic anaerobes which ferment carbohydrates vigorously with the production of acid and gas are:

Clostridium perfringens (*B. welchii*)
Clostridium septicum (*Vibrion septique*)
Clostridium chauvoei (Causes an analogous infection in animals only)
Clostridium novyii (*B. oedematiens*)
Clostridium bifermentans
Clostridium tertium
Clostridium fallax

The more important proteolytic anaerobes which digest protein material with the formation of various amino acids and sulfur compounds which blacken the cultures are:

Clostridium sporogenes
Clostridium histolyticum

The characteristics of the most important of the clostridia are given in Table 6.

Clostridium Perfringens (*Bacillus welchii*) (*Welch, 1891*). This organism, commonly termed the "gas bacillus," was first isolated from gas gangrene cases and received the name *Bacillus aerogenes capsulatus*. Fraenkel gave the name *B. phlegmonis emphysematosae* to the same organism which he also obtained from similar lesions. The name *B. perfringens* was used by the French. This is the cause of foamy liver, and during World War I it was the most common cause of gas gangrene (75 per cent), although nearly always associated with other organisms, especially *Cl. sporogenes* and certain aerobes. In World War II it was less common. It has always been responsible for most cases in civilians. Apparently only certain markedly virulent strains of the gas bacillus can produce gas gangrene when present alone and then only when much necrotized tissue is present in the wound. It is this requirement which led to the development of the surgical procedure of débridement.

Morphology and Cultural Characteristics. The gas bacillus is a large ($5 \times 1.3\mu$), nonmotile, Gram-positive anaerobe. It forms oval, centrally placed spores, which but slightly distend the rod. These spores are not formed in the wound, and it is difficult to bring about their development in ordinary culture media. Protein media, such as blood serum

Table 6. CHARACTERISTICS OF IMPORTANT ANAEROBES

	Motility	Spore	Liquefy: Gelatin	Liquefy: Coag. Serum	Milk	Deep Meat or Brain	Glucose	Mannitol	Lactose	Sucrose	Starch	Salicin	NO_3	Indol	H_2S	Toxin
Cl. perfringens	—	Oval, subterm.	+	—	Stormy ferm.	Gas Pink	+	—	+	+	+	—	+	—	+	+
Cl. septicum	+	Oval, subterm.	+	—	AC Gas	Gas Pink	+	—	+	—	—	+	+	—	—	+
Cl. chauvoei	+	Oval, subterm.	+	—	AC Gas	Gas Pink	+	—	+	+	+	—	+	—	+	+
Cl. fallax	+	Oval, subterm.	∓	—	AC Gas ∓	Gas Pink	+	—	∓		+					weak +
Cl. tertium	+	Oval, term.	—	—	AC	Gas	+	+	+	+	+		+	—		—
Cl. novyii	+	Oval, subterm.	+	—	AC Gas	Gas Pink	+	—	—	—	+	—	—	—	+	+
Cl. bifermentans	+	Oval, eccentric	+	+	C Digest slow	Black Digest	+		—	—		∓	—	+	+	+
Cl. histolyticum	+	Oval, subterm.	+	+	Digest	Digest Black	—	—	—	—	—	—	—	—	—	weak +
Cl. sporogenes	+	Oval, subterm.	+	+	Digest	Gas Black Digest	+	—	—	—	—	—	—	—	+	—
Cl. tetani	+	Round, term.	+	—	—	Gas	—	—	—	—	—	—	—	∓	—	+
Cl. botulinum A & B	+	Oval, subterm.	+	+	Alk. Digest	Gas Black Digest	+	—	—	—	—	+	—	—	+	+
Cl. botulinum C, D, E	+	Oval, subterm	+		A		+									

or egg, are the most favorable. It is inhibited by the presence of sugar in media. The bacillus shows a capsule in smears from animal tissues or fluids. Some strains exhibit hemolytic power when grown on blood agar. Cooked-meat media are in general best for culturing, but the most characteristic cultural peculiarity is seen when the organism is grown in milk, under anaerobic conditions. There occurs coagulation with disruption of portions of the coagulum into shreds, colored by acid formation, if an indicator is present, and plastered against the sides of the tube. The gas-riddled mass of coagulum remaining floats in a clear whey which has the odor of butyric acid. All the common sugars are fermented with the production of a large amount of gas. Glucose agar to which a little blood is added makes a very favorable medium. Coagulated serum is not liquefied, and indol is not formed in broth. The optimum temperature for growth is 38° C.

Laboratory Diagnosis. The presence of the Welch bacillus can be demonstrated by inoculating material into a tube of milk, heating it to 80° C. for an hour, and incubating it anaerobically for 12 to 18 hours. If this organism is present the so-called "stormy-fermentation" reaction described above may be seen. It is not produced by other anaerobes.

To obtain pure cultures, 3 or 4 ml. of the whey are inoculated into the ear vein of a rabbit. After five minutes the rabbit is killed and the carcass placed in the incubator for six to eight hours. The body should become distended with gas, with crepitation demonstrable in muscles and fascia, and the organism should be obtainable from the foamy liver or the heart blood (Welch-Nuttall test).

At the American Ambulance during World War I a sterile cotton throat applicator was used to obtain the discharge from a wound. Each swab was sent to the laboratory in a tube. A glucose agar tube was boiled for 10 minutes, then quickly cooled to 42° C. The swab was introduced into the melted agar and well rubbed up in it. This agar was then quickly solidified in ice water and put in the incubator. For quick diagnosis the swab, coated with agar, was transferred to the tube from which it was taken and this latter was placed in a larger tube containing pyrogallic acid and sodium hydroxide to obtain anaerobiosis. In four or five hours a smear could be made from this swab and Gram-stained to demonstrate the presence of the gas bacillus.



Cl. perfringens agar culture showing gas formation. (MacNeal.)

Toxin Production. Bull and Pritchett (1917) produced a soluble toxin which causes both tissue necrosis and hemolysis. They found that all strains produced the toxin, but that the amount depended upon the virulence of the strain. Virulence could be increased by animal passage. By the injection of animals with this toxin they have produced an antitoxin. This antitoxin gives complete protection in animals, and has given favorable results in cases of gas gangrene in man.

Organisms resembling *Cl. perfringens*, and producing related toxins, have been found in animals, especially in sheep. Wilsdon (1931) has suggested that they be considered as types of *Cl. perfringens*: type A, the organism of gas gangrene; types B (Dalling, 1925),

C (McEwen, 1930), and D (Bennetts, 1932) those of animals. As a rule, however, type A, which is found in human cases only, is called *Cl. perfringens,* and the other types are considered as separate species. An antitoxin for type B usually protects against all four toxins, but an antitoxin for type A protects only against toxin A. There is more or less cross protection among the other types. The toxin of *Cl. perfringens* is very complex. There seems to be no definite incubation period.

Occurrence. The gas bacillus is common in fertilized soils and in the feces of man and other animals. As previously mentioned, it is a common cause of gas gangrene in war wounds, and is responsible for nearly all cases of gas gangrene occurring in civilians.

The gas bacillus may also cause uterine infections, particularly those resulting from abortions. It may invade the blood immediately before death, and give rise to the gas bubbles in the organs and tissues which are sometimes seen at autopsies.

Kendall has called attention to the importance of this organism in a certain proportion of cases of summer diarrhea of infants. (See Examination of the Feces. Chap. 39.)

The active gas-production by this organism has led to its occasional use as a "bread starter" and as an indicator of fecal pollution in water supplies.

Clostridium Septicum (*Vibrion septique*) (*Pasteur, 1877*). This organism was isolated by Pasteur from an animal supposed to have died of anthrax. Later, Koch isolated an anaerobe from garden soil which he named *B. oedematis maligni* although he regarded it as identical with Pasteur's *Vibrion*. Although neither description was complete enough to allow identification today, the original *V. septique* of Pasteur has been preserved so that it can be adopted as a "type strain."

Morphology and Cultural Characteristics. *Vibrion septique* is a rather narrow bacillus with rounded ends measuring on the average $6 \times 0.4\mu$. It is motile, and, in a fresh specimen of the blood of a guinea pig dying of the infection, appears in long undulating chains which move among the blood cells "as serpents in the grass" (Pasteur). In wet preparations from the liver of the guinea pig, these serpent-like forms are common. The bacillus forms oval spores which usually lie between the center and the end, but rather characteristic is the formation of a spindle-shaped organism with a large central spore. Sporulation does not seem to take place in the body of the host. The organism is Gram-positive. It ferments milk with gas production, but less actively than does the Welch bacillus. It has little proteolytic action; not liquefying but only softening gelatin, and not acting on blood serum. It ferments glucose and lactose, but not saccharose. The colonies are filamentous and spreading. The odor of culture is sour or rancid—not foul.

Pathogenicity. The organism has been occasionally found in war wounds, usually in association with other anaerobes. It produces a soluble toxin which causes edema and necrosis of tissues and contains a hemolysin. This toxin, like that of the gas bacillus, seems not to require a period of incubation, but excites the formation of an antitoxin. A rather large dose is required to kill an animal. The lesions of animals dying of the infection resemble those produced by the gas bacillus, but the *Vibrion* may be distinguished from the gas bacillus by its motility. In man the infection has been noted only in war wounds, but in herbivorous animals it may occur with or without wounds. *Clostridium septicum* has been isolated from milk.

Clostridium Chauvoei (*Bacillus chauvoei*). This is an anaerobic spore-bearer, called the bacillus of symptomatic anthrax, blackleg, or quarter-evil, which causes a rapidly developing emphysematous swelling, with a dark color, of the thighs. It has bulging, slightly oval spores at one end, but they are not distinctly terminal as are tetanus spores. It affects sheep and cattle but not man. It is a soil organism like those of tetanus and gas gangrene.

It is difficult to separate this organism from *Cl. septicum,* but as the latter alone is

concerned in human infections this difficulty is important only for veterinarians. Robertson gives as differentiating points the fermentation of salicin but not saccharose by *Cl. septicum* while *Cl. chauvoei* ferments saccharose but not salicin. *Cl. chauvoei* does not exhibit the gliding-serpent chains which are such a feature of fresh liver emulsion and blood preparations from guinea pigs infected with *Cl. septicum*. Many have reported *Cl. chauvoei* as Gram-negative. The thermolabile soluble toxin produced by this organism is relatively weak.

Clostridium Novyii (*Bacillus oedematiens*). This bacillus is quite large ($6 \times 1\mu$) and is usually stated to be nonmotile, although some authorities report young cultures as motile. It is more strictly anaerobic than the other pathogenic anaerobes. It liquefies gelatin. The spores, which form readily, are large (1.5μ) and located eccentrically. It produces a soluble toxin against which an antitoxin has been produced. Injection of the toxin (0.05 ml.) kills guinea pigs in one or two days.

This organism was a rather common one in the wounds of World War I. It causes less tissue destruction than *Cl. perfringens,* but forms quite a potent toxin. Injected into the muscular tissues of a guinea pig it produces a gelatinous necrosis with only slight gas formation. Very small amounts of the toxin are fatal for guinea pigs.

Clostridium Bifermentans (*B. sordelli*). This microörganism is also sometimes involved in gas gangrene. It is a large, sluggishly motile bacillus which produces acid and gas only from dextrose. All pathogenic strains produce a fairly strong soluble toxin.

Therapy of Anaerobic Infections of War Wounds. Antitoxin used in therapy of gas gangrene may be monovalent or polyvalent, is usually refined and concentrated, and is standardized by mouse-protection methods recommended by the National Institute of Health, Washington, D.C. Monovalent antitoxin is sometimes prepared against *Clostridium perfringens* (*B. welchii*), as this organism is the principal one involved in civilian cases. But in war wounds other members of this group are often present, and a trivalent antitoxin is in most common use. This is prepared by immunizing horses separately with the toxins of *Cl. perfringens* (*B. welchii*), *Cl. septicum* (*Vibrion septique*), and *Cl. novyii* (*B. oedematiens*), and then mixing them in the desired unitage. Sometimes tetanus antitoxin is also included, and occasionally those for *Cl. bifermentans* and *Cl. histolyticum*. If serum therapy is to be used successfully in gas gangrene it should be used early, and it should cover the toxin-producing species involved. The determination of the species present can be quite time-consuming. Henry (1917) has suggested a method for a quick identification of the important saccharolytic anaerobes in wounds, *Cl. perfringens, Cl. septicum,* and *Cl. novyii*. The material is inoculated into a cooked-meat medium, and from this into a tube of milk. If the stormy fermentation occurs *Cl. perfringens* is present. At the same time some of the culture is inoculated into two guinea pigs, one of which has received *Cl. septicum* and *Cl. perfringens* antitoxin, and the other *Cl. novyii* and *Cl. perfringens* antitoxin. If the first pig dies it indicates that some organism other than *Cl. septicum* or *Cl. perfringens* is present, and this is most often the *Cl. novyii*. This assumption is confirmed if the second pig survives. If the second pig alone dies one may conclude, by the same reasoning, that *Cl. septicum* is responsible. If both pigs die either both *Cl. novyii* and *Cl. septicum* or some other anaerobes are probably present, and identification must be made by cultural methods.

Toxoids for active immunization against the toxins of the gas gangrene organisms have been developed. Their use is still in an experimental stage.

Local treatment of lacerated wounds consists in immediate débridement in order to make conditions unfavorable for the growth of bacteria, in particular these anaerobes. Before closure is attempted smears from the wound should show few, if any, anaerobes, and possibility of the presence of hemolytic streptococci should be excluded by cultures on blood agar.

During World War II both tetanus and gas gangrene have been remarkably rare. The use of sulfonamides, both locally and systemically, has given favorable results.

Proteolytic Anaerobes of War Wounds: Clostridium Sporogenes (*Bacillus sporogenes*). This organism differs from the gas bacillus and the *Cl. septicum* in being actively proteolytic whereas the two latter act on carbohydrates rather than on proteins. During World War I *Cl. sporogenes* was frequently encountered in war wounds and was regarded as the main cause of their foul odor. Like the gas bacillus, it is often found in human or animal feces and in fertilized soils.

Cl. sporogenes is an actively motile, Gram-positive bacillus with rounded ends ($5 \times 0.8\mu$); it liquefies gelatin and digests blood serum. It does not seem to be pathogenic, but its proteolytic enzymes decompose the tissues killed by the mechanical injury produced by the gas-forming clostridia. It does not produce an exotoxin.

It is a common contaminant of other anaerobic cultures, and since its spores have great power of resistance it is difficult to separate it from organisms that one desires to obtain in pure culture. Barber's single-cell technic is valuable in effecting separation.

Clostridium Histolyticum (*Bacillus histolyticus*). This organism, like *Cl. sporogenes,* has marked proteolytic power. In fact, this faculty is so great that the intramuscular injection of cultures into guinea pigs may bring about digestion of the muscles down to the bone within a few hours—without, however, having much effect on the health of the animal.

It is a motile bacillus, about $4 \times 0.6\mu$, and forms oval spores which tend to be eccentric. Cooked-meat media as well as blood serum are rapidly digested. Although no toxin is produced as a rule, toxic materials can be prepared which are lethal to animals when given intravenously. This organism is micro-aerophilic, although it grows best under strict anaerobic conditions.

Tetanus

In 1884, Carlo and Rattone caused tetanus in rabbits by inoculating pus from a human case. Nicolaier, in 1885, produced tetanus by injecting garden soil into animals. Also in 1885, Kitasato obtained pure cultures by incubating tetanus-containing material for 48 hours, then heating at 80° C. for one hour (thus killing nonsporing organisms), and culturing anaerobically; he produced tetanus in animals by inoculation with this culture.

Clostridium Tetani (*B. tetani*). The tetanus bacillus is common in the feces of horses and cattle; hence its frequency in cultivated soils. The horse is the most susceptible animal, next the guinea pig, then the mouse. The infection occurs in cattle and sheep. Dogs are much less susceptible. Fowls are practically immune. Man is highly susceptible.

Morphology. *Cl. tetani* is a long slender bacillus ($4 \times 0.4\mu$). It is slightly motile and forms a large (1.3μ) drumstick spore. Since certain other anaerobes, such as *Cl. tertium,* form similar terminal spores, a diagnosis of tetanus cannot be made by this finding alone. The organism is Gram-positive, but degenerating forms in old cultures may lose this characteristic.

Cultural Characteristics. The tetanus bacillus grows anaerobically on ordinary media. The addition of glucose or fresh sterile tissue (either acts as a reducing agent) to the culture facilitates its growth, especially in the absence of complete anaerobiosis. Colonies on agar plates are small, and are surrounded by a network of matted filaments. In stab cultures in glucose agar this anaerobe shows as an inverted pine-tree growth. It liquefies gelatin very slowly. Milk is not coagulated. None of the carbohydrates are fermented. Cultures have a slight odor of putrefaction.

Viability. The spores may remain viable in the soil for years. They are very resistant to heat and ordinary antiseptics. They may withstand boiling for an hour or longer, but are usually killed within five minutes in the autoclave.

Variation. Motile and nonmotile variants, and smooth- and rough-colony strains have been described.

Types. At least 10 types of tetanus bacilli have been differentiated by agglutination and agglutinin absorption tests. However, there does not seem to be any type specificity in the toxin, so that an antitoxin made from type 1 will protect against the toxins of the other types. A good summary of serologic studies of the tetanus organisms has been made by Fildes (1929).

Toxin. The tetanus bacillus produces a soluble toxin. It is one of the most powerful poisons known and it is estimated that 0.0002 Gm. is fatal for man. It is said to be 20 times as poisonous as dried cobra venom. It can be prepared by growing the bacilli anaerobically in broth, and filtering after 10 to 14 days. There are, in fact, two toxins: *tetanospasmin* which damages the tissues of the nervous system, and *tetanolysin,* of minor importance, which causes hemolysis of red blood cells. That the disease is due to the toxin is shown not only by the experimental production of tetanus by means of toxic filtrates, but also by the fact that if spores are carefully freed of all toxin by washing, and then introduced, they do not cause tetanus but are phagocyted. When the toxin is injected into susceptible animals death occurs in 12 to 24 hours with typical symptoms of tetanus.

The route by which the toxin reaches the nervous system has been the subject of extensive investigation. It has been believed that the toxin produced locally by the growth of the bacilli was absorbed by the local motor end-plates and traveled directly to the central nervous system by way of the axis cylinders. However, Abel (1934) has published evidence to show that the toxin reaches the central nervous system by way of the blood stream.

The Disease in Man. Tetanus is caused by the action on the central nervous system of a toxin produced by the growth of tetanus bacilli in a deep, penetrating wound. Such wounds, especially when there is much tissue destruction, afford a suitable anaerobic environment. The presence of other bacteria in the wound favors the growth of tetanus bacilli, especially the presence of *Cl. perfringens,* which is frequently associated, and which creates a favorable soil by causing extensive tissue destruction. The spores germinate readily only in an oxygen tension which is lower than that in normal tissues. Tetanus has occurred following childbirth or abortions, and in the newborn from infection of the umbilicus. It has occurred after surgical operations from insufficiently sterilized catgut or other supplies, and following vaccination and hypodermic injections.

After infection of a wound there is a definite incubation period before the first symptoms develop—usually from 5 to 10 days in the acute cases, although it may be much longer. As a rule, the shorter the incubation period, the more severe is the disease. The muscle spasms develop first, as a rule, in the neck and jaw muscles and the muscles around the mouth, giving the characteristic "risus sardonicus." Later the muscles of the trunk and back become affected. The muscle spasms are associated with violent pain, dyspnea, and difficulty in swallowing.

In tetanus, as in diphtheria, the bacilli remain localized in the lesion, and the injury to other organs is caused by the toxin which is absorbed from the wound. However, spores have been found in the regional lymph nodes, and also in the liver and spleen in fatal cases.

The term "chronic tetanus" has been applied to atypical cases developing

slowly, with a relatively mild course. Cases may occur several months after injury or operation, especially with low-grade bone infections.

Bacteriologic Examination of a Wound. Tetanus bacilli can rarely be demonstrated in films made directly from the wound. Animal inoculation is more dependable than cultural methods.

The wound should be curetted and some of the tissue fragments inserted into a pocket in the subcutaneous tissue of the thigh of a guinea pig. The remainder may be inoculated into glucose agar stabs or into a tube of deep meat medium. In these cultures the development of a foul, sour odor is suggestive. Films from such cultures frequently show the drumstick spores. If these are found, an emulsion of the growth is heated to 80° C. for one-half hour to kill nonsporing bacteria. A deep tube of glucose agar, melted and cooled (to 42 to 45° C.), is inoculated with the material which is then cultivated anaerobically. Isolated colonies may thus be obtained.

The filtrate from cultures, even when mixed, can be inoculated into animals (guinea pigs, rats, or mice) to demonstrate the presence of the toxin. This is the most reliable method for demonstrating the presence of tetanus bacilli. A control animal inoculated with the filtrate together with antitetanic serum should be protected.

Antitoxin. The antitoxin is produced by injecting horses with increasing doses of tetanus toxin, at first adding sufficient antitoxin to neutralize it. A high degree of immunity to the toxin is developed. The method of standardization established by law in the U. S. is based on the work of Rosenau and Anderson of the U. S. Public Health Service. The antitoxin unit was originally defined as 10 times the minimal amount of serum necessary to protect a 350-Gm. guinea pig for 96 hours from 100 M.L.D. of a standard toxin. One standard antitoxin unit equals two of the international standard units. Standard antitoxin can be obtained from the National Institute of Health, by means of which others can determine the strength of their own toxin, and indirectly of their antitoxin.

Preventive Measures: Passive Immunization. The practical value of tetanus antitoxin is largely limited to its use as a prophylactic measure. Its effectiveness for this purpose, if properly used, has been demonstrated conclusively. To nonimmunized persons who have sustained any deep injury which may be contaminated by soil or dirt, especially if there is laceration of tissue, it should be given immediately in doses of 1500 units. As the immunity from a single injection lasts only about 10 days, a second dose will often be required at the termination of this period if the wound has not healed. It is particularly important to repeat the injection prior to subsequent operations on the infected area, otherwise surgical intervention may provoke an acute attack of tetanus. Anaphylactic reactions can be obviated by a preliminary desensitizing dose. (See section on hypersensitiveness.)

Active Immunization. By incubating tetanus toxin with 0.3 per cent formaldehyde for several months, its poisonous properties can be destroyed without affecting the antigenic powers. With such tetanus toxoid, or anatoxin (Ramon), it has been possible to immunize animals actively to tetanus, and to stimulate the production of substantial amounts of antitoxin in man. Bergey (1934) found that alum-precipitated toxoid was more antigenic and gave little reaction. The development of tetanus toxoid has been an important step in the prevention of tetanus. Since the beginning of World War II the toxoid has been used to vaccinate members of the armed forces of the United States as well as those of other countries. The U. S. Army has used the plain toxoid, giving three

injections of 1 ml. each three to four weeks apart, with a "booster" injection of 1 ml. at the end of a year. The U. S. Navy has adopted the alum-precipitated toxoid, giving two injections more widely spaced. Results obtained by active immunization with tetanus toxoid have been excellent. The use of tetanus antitoxin prophylactically has been reserved for those who have not been actively immunized.

Serum Treatment. Serum treatment after symptoms have developed is far less efficacious than that of diphtheria, largely because the toxin has already become fixed to the nerve tissues. The nerve cells have a greater affinity for the toxin than has the antitoxin, and when once injured do not recover as readily as other body cells. The mortality of untreated patients in whom tetanus has developed within 10 days following injury is over 80 per cent. Serum treatment has little effect if the incubation period has been less than five days, but it has been reported to lessen the mortality substantially when the incubation period has been 5 to 10 days or longer (to 30 per cent according to Courtois-Suffit and Giroux (1918) and to 52 per cent according to Wainwright (1926)). Simple subcutaneous injections are useless. The serum must be given intravenously, repeatedly, in doses of 20,000 units. Some advise a total of up to 100,000 units the first day. It is also essential that the wound be excised or thoroughly cleaned of all dirt and necrotic tissue and that the spasms be controlled by powerful sedatives such as avertin (Teichmann, 1932) or sodium amytal.

Botulism

Clostridium Botulinum (*B. botulinus*) (*Van Ermengem, 1896*). This organism produces botulism, a form of food poisoning. It is a spore-bearing anaerobe, and must not be confused with the salmonellas, some species of which may also be associated with food poisoning. The organism is found in virgin as well as in cultivated soil, in manure, and on vegetables, fruits, etc.

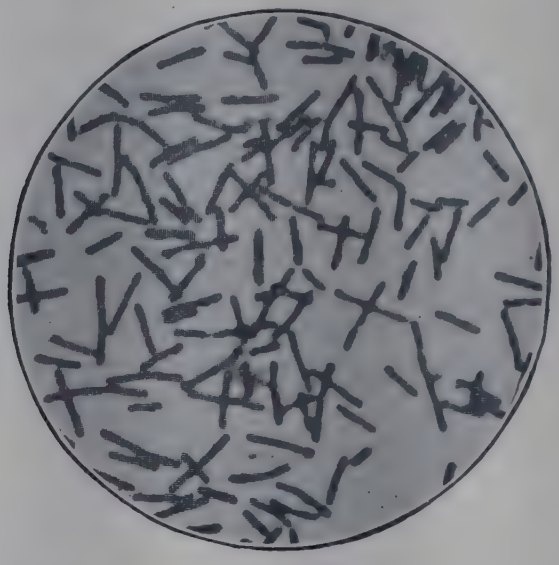
Morphology and Cultural Characteristics. *Cl. botulinum* is a large, coarse bacillus 5 to $7\mu \times 1\mu$, occurring singly or in very short chains. It is slightly motile and stains by the Gram method. When sporulating the spore is near the end. This microörganism grows anaerobically on the ordinary media. It does not coagulate milk, which is a favorable medium. It ferments glucose with the production of a great deal of gas. Gelatin is usually liquefied. Cultures have a sour, rancid odor. The bacillus grows well at room temperature, less well at body temperature. It is heterogeneous biochemically as well as serologically. Five types have been recognized. All liquefy gelatin and ferment dextrose, levulose, maltose, and glycerol, but there is considerable variation among the strains within the types in regard to other biochemic characteristics. Type A and, to a certain extent, type B are proteolytic, whereas types C, D, and E are not.

Toxin. In contaminated foods and in cultures a powerful exotoxin is formed which is responsible for the symptoms produced. It is so potent that as little as 0.000001 ml. may kill a guinea pig. This toxin is destroyed easily by heating to 80° C. for one-half hour, or by boiling for 10 minutes; it is unlike the sporulating bacillus which may survive boiling for some hours.

Two types of *Cl. botulinum*, A and B, are responsible for most cases of botulism in human beings, and recently type E has been found involved (Hazen, 1942). Types C and D have not been found in human cases; C has been extensively involved in affections in ducks, and D has been found chiefly in ruminants in Africa. In chickens, type A toxin produces a paralysis with weakness of the neck (the so-called limber neck), while type B toxin may produce no symptoms. The toxins of the various types differ, and the antitoxin of one type fails to neutralize the toxins of the other types.

Source of the Infection. In Europe the chief sources of infection have been contaminated meat, especially ham and sausage. In this country the outbreaks reported

have resulted from eating canned vegetables, fruits, ripe olives, and cheese. Commercial canned products are more likely to be safe than food preserved in the household. It is important to remember that the canned foods may not appear decomposed in any way, and yet may contain the bacilli and their toxin. Meat becomes infected only after the animal has been slaughtered, while fruits and vegetables are already contaminated from soil, and possibly by insects. Since the toxin is destroyed by moderate heating, cooking food immediately before eating removes danger of botulism.



Bacillus of botulism. (Kolle and Wassermann.)

Infection in Man. Clinical symptoms in man are due to the absorption of the toxin, which, unlike those of diphtheria and tetanus, is elaborated outside of the body and ingested preformed in the food. It is not destroyed in the alimentary tract but is absorbed directly from the stomach and duodenum. The specific manifestations do not appear for from 12 to 36 hours or longer, although gastrointestinal disturbances may occur earlier if large amounts have been ingested. The characteristic symptoms are oculomotor paralyses with diplopia and mydriasis, and paralysis of the pharyngeal muscles with difficulty in swallowing. In fatal cases there are cardiac and respiratory failures from involvement of the medullary centers. There is no fever, and the mentality remains clear throughout. Although *Cl. botulinum* may be found in the gastrointestinal tract, and in fatal cases in the spleen, there is no danger of infection from one individual to another, as in the case of food poisoning due to salmonella infection. The toxin is ordinarily swallowed and not elaborated in the gastrointestinal tract.

Examination of Food. To ascertain the presence of the toxin in the food, filtered extracts or supernatant fluid from centrifuged emulsions of the suspected food may be injected intraperitoneally into a guinea pig or mouse. If the toxin is present characteristic bulbar and pupillary symptoms will result, with death by cardiac or respiratory failure. Another animal should be injected together with the antitoxin as a control, and a third may be given some of the material that has been boiled for 10 minutes. Cultures can be made by heating an emulsion of the food at 60° C. for an hour to destroy nonsporing bacteria, and making anaerobic shake cultures in glucose agar. These should be incubated anaerobically at room temperature

in the dark. Filtrates or supernatant fluid from broth cultures can be tested for the presence of the toxin by animal inoculation. Feeding chickens with suspected material has been used to differentiate the types though this may be done more accurately by giving the specific type antitoxins.

Serum Treatment. Potent antitoxins can be prepared against the botulinus toxins. Under experimental conditions they have been highly successful, but they are much less useful in clinical cases. This is because the antitoxin can neutralize only the free-circulating toxin, and the combination of the toxin with the nerve tissue has already occurred by the time symptoms develop. Nevertheless, whenever possible, the antitoxin (combined for types A and B, and possibly E) should be administered, for it is the only specific treatment known, and it is always possible that uncombined toxin may still be present.

Active Immunization. Velicanov (1936) used a formol toxoid (types A and B) to immunize a small number of volunteers and laboratory personnel. More recently Reames and his coworkers (1947) have used both fluid and alum precipitated toxoids to vaccinate a group of persons working with *Cl. botulinum*. They succeeded in maintaining a good antitoxin titer (A and B) in these people by giving booster injections every six months.

Bennetts and Hall (1938) have used a formol toxoid on a small scale to immunize cattle and sheep in Australia.

CHAPTER 4

Mycobacteria, Actinomyces and Nocardia, Corynebacteria, and Other Gram-positive Bacteria

Key and Notes

General Features. Having branching characteristics; showing parallelism, branching, curving forms, V-shapes, clubbing at ends, segmental staining, etc. Gram-positive.

Order II. *Actinomycetales*. Cultures more like molds.
Family I. Mycobacteriaceae. Very slight branching.
Genus: *Mycobacterium*. Acid-fast. Cultures usually more or less wrinkled and dry.
I. Grow rapidly on ordinary media at room temperature. Typical species are:
1. *Mycobacterium phlei*. (Timothy-grass bacillus of Moeller.)
2. *Mycobacterium lacticola* (*M. smegmatis*). (The "smegma" bacillus.)
II. Grow only at body temperature and after several weeks of incubation. Primary isolation requires such culture media as solidified blood serum, glycerin agar, glycerin potato, and egg media.
A. Cultures fairly moist, luxuriant, and flat. Optimum temperature 43° C.
1. *Mycobacterium avium*. (Bacillus of avian tuberculosis.)
B. Cultures scanty, wrinkled, and dry. Appear in 10 to 14 days. Optimum temperature 38° C. Smear from organs of inoculated guinea pig shows few bacilli. Less virulent for rabbits.
1. *Mycobacterium tuberculosis* var. *hominis*. (Bacillus of human tuberculosis.)
C. Cultures as above, but even more scanty. Smear from organs of guinea pig shows many bacilli.
1. *Mycobacterium tuberculosis* var. *bovis*. (Bovine tubercle bacilli.)
III. Primary isolation requires growth factor from heat-killed acid-fast bacilli.
1. Organism of Johne's disease.
IV. Are not cultivable by ordinary methods.
1. *Mycobacterium leprae* (*B. leprae*). Found in man chiefly in nasal mucus and in juice from lepra tubercles. Less often in nerve leprosy.
2. *Mycobacterium lepraemurium*. (Bacillus of rat leprosy.) Indistinguishable from *M. leprae* except by inoculation into young rats.
Family II. Actinomycetaceae. Filamentous and branching, forming mycelia which break up into fragments which may function as conidia.
Genus: *Actinomyces*. No aerial hyphae. Anaerobic. Not acid-fast.
1. *Actinomyces bovis* (*A. israeli*). Cause of actinomycosis.
Genus: *Nocardia*. Short aerial hyphae. Aerobic. Some species acid-fast.
1. *Nocardia madurae*. Cause of mycetoma. Not acid-fast.
2. *Nocardia asteroides*. Acid-fast.
Order I. *Eubacteriales*. The "true" bacteria.
Family VIII. Corynebacteriaceae. Colonies more flat and moist like other bacteria.
Genus: *Corynebacterium*.
1. *Corynebacterium pseudodiphtheriticum* (*B. hoffmanni*). Luxuriant growth on ordinary media. Colonies often yellow to brownish. Short, thick, and stain uniformly.

2. *Corynebacterium diphtheriae*. Moderate growth on ordinary media. Best media are blood serum (Loeffler's) or blood agar. Has metachromatic granules at poles.
3. *Corynebacterium xerose*. Scanty and slow growth on nutrient media.

Genus: *Listeria*. Very small Gram-positive rods with arrangement similar to that of diphtheroids. Motile.

1. *Listeria monocytogenes*.

Genus: *Erysipelothrix*. Rod-shaped organisms with a tendency to form filaments which may branch.

1. *Erysipelothrix rhusiopathiae*. Cause of swine erysipelas.

Other Gram-positive bacteria:

Family VII. Lactobacteriaceae. Ferment carbohydrates, producing lactic acid.

Tribe II. Lactobacilleae. Rods singly, in pairs, or filaments.

Genus: *Lactobacillus*. Rods long and slender; micro-aerophilic or anaerobic; surface growth on media—delicate or poor.

1. *L. acidophilus*.
2. *L. bifidus*.
3. *L. bulgaricus*.
4. *L. casei*.
5. *L. arabinosus*.
6. *L. fermenti*.

Mycobacteria

The mycobacteria comprise a large group of strictly aerobic bacilli which possess abundant and highly characteristic lipids. These lipids modify the reaction of the bacilli to stains, affect their growth rate and their permeability to solutions or disinfectants, interfere with their destruction in phagocytic cells, and contribute to the typical pathology of diseases such as tuberculosis and leprosy.

The single criterion which determines the inclusion of bacteria in this group is the property of "acid-fastness"; i.e., resistance to decolorization by acids or acid-alcohol after the bacilli have once been penetrated by certain aniline dyes. This property is largely due to unsaponifiable waxes. Among the pathogenic members of the group phospholipins play an important part in producing the epithelioid-cell type of pathologic reaction, while the combined qualities of slow growth and resistance to phagocytic digestion may contribute to the chronic persistent nature of the mycobacterial infections.

On the basis of the general character of their growth habits or requirements, this great group of bacteria is divided into four subgroups which correspond closely with the sources from which they are most frequently recovered and with their disease-producing potentialities. These groups are indicated in the foregoing key and in Table 7, on p. 77. The first group is capable of growth on ordinary media, and usually at a wide range of temperatures. It contains primarily the saprophytic acid-fast organisms of soil and water. Some strains cause tuberculosis in cold-blooded animals and others occur as parasites in organs, lymph nodes, and skin lesions in animals, or in leprous nodules. The second group contains the tubercle bacilli of warm-blooded animals. These have more complex

growth requirements for primary isolation, and grow only at or near body temperatures. Their pathogenic potentialities are not confined to a single animal species. Owing to their remarkable antigenic properties and a violently hypersensitive response in most hosts, they achieve great destructiveness. The third group requires a growth factor which can be obtained from the bodies of other killed acid-fast bacteria. It produces less destructive lesions, involves a more limited group of tissues, and is infectious for a smaller number of animal species. The growth requirements of the fourth group have not yet been ascertained. Its two species illustrate the maximal degree of indolent infection and host specificity.

Saprophytic, Cold-blooded Animal Types, and Parasitic Mycobacteria

In view of the widespread distribution of the members of this subgroup and their remarkable adaptability to different temperatures and conditions of growth, they have repeatedly been confused with the truly pathogenic types. The medical importance of this fact is illustrated by the necessity of accepting the smegma bacillus as one long known to soil and dairy bacteriologists under the name of *Mycobacterium lacticola* (Bergey, 1939) and by the indistinguishability of the parasitic strains in animal lesions and leprous nodules from those which occur commonly in soil and nature (Gordon, 1937).

Those who have studied tuberculosis in cold-blooded animals likewise have been confused by differences in pathogenicity, ranging from the occurrence of acid-fast bacteria in water or algae or in the external slime of fish, through apparently innocuous parasites in the organs and, finally, to true tubercle production by strains which can reproduce the infection experimentally. Although many of the microörganisms causing true tuberculous infection in cold-blooded animals have a temperature range below the body temperature of birds and mammals, Aronson found a strain from the salt-water croaker (fish) which was pathogenic for mice, pigeons, and frogs, but not for guinea pigs.

The fundamental point for the medical bacteriologist is the recognition that among these organisms there are both saprophytic and parasitic strains which on primary isolation may grow slowly and may develop pigmentation only late or in subculture. Their colonies may correspond more or less with those of different types of tubercle bacilli, being either moist and irregularly flat like those of the avian type or dry and wrinkled like those of the mammalian types. The smegma bacillus is one widely recognized example of a member of a saprophytic or parasitic group which may complicate the laboratory diagnosis of true mycobacterial infections. Other members, less well known, are probably responsible for a number of errors in interpretation.

These nonpathogens differ from the tubercle bacillus in five important essentials:

1. They grow readily on any media.
2. They show more or less abundant growth, or colonies within two to seven days.
3. They have no pathogenicity for guinea pigs when inoculated subcutaneously.

Table 7

CLASSIFICATION AND CHARACTERIZATION OF THE MYCOBACTERIA*

General Character of Growth	*Natural Habitat and Other Sources from Which Isolated*	Surv.† 60° C.	Grow† 47° C.	*Representative or Type Species*	*Experimental Pathogenicity*
Grow rapidly (2 to 7 days) on ordinary media at room temperature; usually pigmented; often of smooth-colony type				(*Mycobacterium*)	Nonpathogenic for warm-blooded animals; although many cause suppuration. Local injection may produce lesions which may be mistaken for true tubercles or leprous granulomas. Large doses intravenously often prove fatal
	Soil, timothy grass, animal skin lesions	+	+	*phlei* (timothy grass)	
	Soil, tubercles of cold-blooded animals, animal lymph nodes or skin, leprosy	—	—	*marinum* (fish) *ranae* (frog) *thamnopheus* (snake) "leprae" (human)	
	Soil, manure, butter, nasal secretions, smegma, leprosy	—	+	*butyricum* (butter) "leprae" (human) *lacticola* (smegma)	
					FOWLS / RABBITS / CALVES / G. PIGS
Primary isolation requires blood, egg yolk, or potato media and several weeks of incubation at body temperatures	Avian tuberculosis, more rarely from cattle, pigs and sheep			*tuberculosis* (avium)	+++ / ++ / — / —
	Human tuberculosis, also from monkeys, pigs, dogs, and parrots			*tuberculosis* (hominis)	— / + / — / +++
	Bovine tuberculosis, more rarely from pigs, man, horses, dogs, cats, sheep			*tuberculosis* (bovis)	— / +++ / +++ / +++
Primary isolation requires growth factor from heat-killed acid-fast bacteria	Johne's disease (chronic enteritis of cattle and sheep)			*johnei* or *paratuberculosis*	— / — / + / —
Do not multiply on bacteriologic media	Human leprosy			*leprae*	Not experimentally transmissible
	Rat leprosy			*leprae-murium*	May be passed through rats and some strains of mice

*Dr. John Hanks supplied this table and much of the material in this discussion of the mycobacteria.
†Survival at 60° C. for one hour; growth over wide temperature range, including 47° C.

4. They do not require body temperature for development, but grow at room temperature or a little above.

Mycobacterium Lacticola (*Bacillus smegmatis*). This acid-fast organism is chiefly important because it may be found in uncatheterized urine and be reported as the tubercle bacillus.

Although they may have the appearance of typical tubercle bacilli, these bacilli show a greater tendency to appear in clumps, as do lepra bacilli. It is usually stated that they decolorize easily in alcohol, but smegma organisms have been found in urine that are as resistant to acid-alcohol as are tubercle bacilli. Such organisms are found in preputial and vulvar secretions, and this accounts for their presence in urine. The Lustgarten bacillus, reported in 1884 as the cause of syphilis, was probably a smegma bacillus. Differentiation is always accomplished by inoculation of a guinea pig, for which, as for man, the organism is nonpathogenic.

Tubercle Bacilli

The three types of tubercle bacilli have been defined in Table 7, where both the usual and the less commonly infected species of animals are indicated. At the usual temperature of 37° C., and on any chosen medium, bacilli of the avian type produce as much growth in two weeks as may be expected from the human type in three, or the bovine type in four weeks. Though morphologic differences between the types are usually described, they are not sufficiently conspicuous or constant to aid in differentiation. Cultural characteristics, quantitative tuberculin tests, or specialized serologic methods are capable of distinguishing the avian type from the mammalian types (human or bovine), but are of little value in differentiating the mammalian types from each other. In actual practice, the differentiation of all three types is dependent on animal inoculation, employing chickens, rabbits, and guinea pigs as the test animals. Owing to the fact that large or uncontrolled dosages may in some instances overcome normal resistance if given intravenously or may form nonprogressive tubercles if given locally, and to the occasional occurrence of atypical strains, known amounts of culture (in the range of 0.1 mg. moist weight) should be injected subcutaneously. The possibility of spontaneous tuberculosis or of occasional abnormal susceptibility in a single animal makes it desirable to include more than one animal of each species in such tests. The results to be expected on the basis of the susceptibility of these animals to the three types of tubercle bacilli have been indicated in Table 7.

Human Type. Mycobacterium Tuberculosis var. hominis (*Bacillus tuberculosis*) (*Koch, 1882*). This type of tubercle bacillus was the first to be studied. It may be taken as a prototype for a general description of the morphology, staining, and other characteristics of tubercle and acid-fast bacilli.

Morphology and Staining. *Mycobacterium tuberculosis* var. *hominis* is a rather long, slender rod, 2 to 5 × 0.3μ, straight or slightly curved, with rounded ends. It is found singly or in clumps in which the bacilli tend to lie parallel or form an acute angle with one another. Many of the organisms show a characteristic beaded appearance in stained films. The bacillus is nonmotile, and Gram-positive.

Owing to their high lipid and wax content, acid-fast bacteria require heating or prolonged application of powerful stains such as Ziehl-Neelsen's carbolfuchsin. Once stained, they retain the dye during treatment with decolorizing agents and do not take a counterstain, thus being differentiated from other bacteria which may be present. A combination of the acid-fast and the Gram-staining methods, as recommended by Fontes, serves to demonstrate Gram-positive rods and granules together with the acid-fast forms. (See Staining Methods, Chapter 12.) Much described non-acid-fast, Gram-positive granules in both cultures and lesions, and M. C. Kahn has made a detailed study of the non-acid-fast granules and rods which appear during the early stages of bacillary multiplication. Such observations help to explain the reports of filtrable forms of tubercle bacilli.

Cultures. Detailed descriptions of media for primary isolation of tubercle bacilli will be given in Chapter 11, but it may be noted here that the basic substrates are, or include, serum, blood, egg yolk, and potato. With the partial exception of the bovine type, glycerol is of universal value in stimulating growth. Contrary to popular belief, minimal numbers of tubercle bacilli can initiate growth in liquid media containing diluted blood or serum and small amounts of an ammonium salt, glycerol, and citrate. Growth on all media is slow. Colonies of *M. tuberculosis* var. *hominis* appear on solid media only after two or three weeks and gradually coalesce to form a thin film which later heaps up in convolutions. The growth ordinarily looks rough and is extremely tenacious on the medium. In liquid media it appears like grains of sand or flecks of wax.

Colonies, pellicles, or granules will not suspend uniformly in any solution except in the presence of chloroform, which is a remarkable (though lethal) "declumper" for all acid-fast bacteria.

Inoculum for primary isolation should be spread as widely and evenly as possible, while for stock cultures it is preferable to plant the inoculum in one small spot. After serial subcultivation, large inocula can establish growth on glycerol agar or as pellicles on glycerol broth, or on synthetic media which provide ammonium or asparagin as sources of nitrogen, and glycerol, glucose, or organic acids as carbon sources. Transplants to liquid media for mass cultivation or tuberculin production must be made by floating bits of growth on the surface.



Mycobacterium tuberculosis var. *hominis;* glycerin agar-agar culture, several months old. (Curtis.)

Tubercle bacilli and other acid-fast microörganisms are strictly aerobic. Abundant oxygen, like high concentrations of glycerol, is important in tuberculin production or mass cultivation, but for primary isolations it is a less important consideration than conservation of the moisture in the medium. Cultures remain viable for months if kept moist. Tubercle bacillus cultures produce a distinctive, fruity odor. Classification by the usual biochemic reactions is not feasible, because the utilization of carbohydrates involves their complete oxidation to carbon dioxide and water.

Viability. The special resistance of tubercle bacilli to acids and alkalies is utilized as a means of killing off contaminating microorganisms prior to the primary isolation of

tubercle bacilli from sputum, gastric washings, or urine. Chemical disinfectants as ordinarily applied are unable to kill these bacilli; for example, they survive in 0.5 per cent phenol for 24 hours. The organisms are very resistant to drying, especially in sputum. Their inaccessibility to substances in solution does not prevent their being killed by sunlight nor confer any particular resistance to heat. They are killed at 60° C. in about 20 minutes. In milk they are devitalized during pasteurization by any method which does not permit separation of a cream layer.

Bovine Type. The bovine type of tubercle bacillus grows even more slowly than the human type. This is particularly true of primary isolations, owing to their failure to be benefited by the presence of glycerol in isolation media. Though the ability to utilize glycerol, or to grow on a synthetic medium, is acquired by some laboratory cultures, many of them continue to utilize glycerol so slowly that they develop an alkaline reaction on liquid media. However, this fact, like the shorter and plumper appearance of the stained bacilli, does not provide a reliable means of differentiating them from the human type. Growth of the bovine type is neither wrinkled nor pigmented.

Bacilli of the bovine type, both in the natural course of events and in experimental infections, are the most virulent of the tubercle bacilli. Aside from tuberculosis in cattle, they are responsible for abdominal tuberculosis in children who consume unheated milk from tuberculous cows, and they are frequently recovered from infections of skin, bones, and lymph nodes of both children and adults in countries where bovine tuberculosis is common. Their occurrence in the pulmonary form of tuberculosis, by contrast, is much rarer than might be expected. Infections with the bovine type are less common in the United States than in many other countries, owing to control of bovine tuberculosis in cattle.

On the farm, bovine-type infections are transmitted from cattle to other livestock, most notably to swine, cats, horses, and sheep. They are measurably more virulent than the human type in guinea pigs, rats, and mice while their marked pathogenicity for rabbits affords a means of differentiating these two types.

Intravenous injection of 0.01 to 0.1 mg. of bovine culture into rabbits causes general miliary tuberculosis and death within five weeks. With human cultures in doses of 0.1 to 1.0 mg., similarly injected, the majority of rabbits live for three months or more. Subcutaneous injection of 10 mg. bovine tubercle bacilli causes death in 28 to 101 days. Similar injections of human organisms in doses up to 100 mg. do not kill rabbits after periods of from 94 to 725 days. The duration of life in inoculated guinea pigs is longer with human than with bovine inoculations.

Subcutaneous injection of bovine strains into cats produces generalized tuberculosis, whereas the cat is resistant to human strains thus given. The cat and dog sometimes spontaneously contract human tuberculosis, although experimentally refractory.

Avian Type. The avian type of tubercle bacilli is characterized by the ability to grow at 43° C. Isolated colonies are usually fairly smooth and capable of being emulsified or suspended much more readily than those of the mammalian-type bacilli. Pellicles on liquid media are correspondingly more difficult to establish,

and there is a greater ability to grow below the surface of glycerol broth or synthetic media.

There is no incontrovertible evidence that tubercle bacilli of the avian type can produce infections in human beings. On farms the infection spreads from fowls to hogs and cattle. Fowls may be infected by ingestion and by injection. After ingestion the lesions are chiefly in the alimentary tract; after injection, in the liver, spleen, and lungs. Though less virulent than bovine bacilli for the rabbit, the avian type can produce progressive lesions after either intravenous or subcutaneous injection in this animal. After intravenous injection, grossly recognizable tubercles are often difficult to find, and the rabbit succumbs to the presence of enormous numbers of bacilli in the phagocytic cells of the spleen and the liver (Yersin type of infection).

Atrium of Infection. In the bovine types of human tuberculosis the portal of entry is the alimentary tract. It is believed that the tubercle bacillus may penetrate the intact mucosa of the intestinal tract wihout causing pathologic changes and reach other areas in the body through the lymphatics. In some infections due to human strains the organisms apparently enter the body in the same way from food or milk which has been contaminated directly from human cases. Most of the cases, however, result from the inhalation of infected dust or from droplet infection from patients with open lesions. The resistance of the bacilli to drying, especially in sputum, permits them to be disseminated by the fomites and by dust and dirt.

Whether or not active disease develops after entry of the bacilli into the body depends upon their virulence and upon the resistance of the body to their invasion. It is probable that ordinarily the actual infection occurs in childhood, and that the disease may become active later on, whenever the general resistance is lowered to such an extent that the bacilli are enabled to multiply and invade the tissues. Possibly, by direct contact with an infective individual, one may receive suddenly a sufficiently large number of bacilli to overwhelm the natural resistance. The susceptibility of an individual varies markedly with age and is greatest in the first few years of life.

Pulmonary tuberculosis may be complicated by secondary infections. Streptococci, pneumococci, staphylococci, or influenza bacilli are frequently present in the neighborhood of the tuberculous lesion and can be found in the sputum.

Diagnosis of Tuberculosis. Careful clinical and bacteriologic studies are required in making a diagnosis and in ascertaining the extent of the disease and its dangers to other members of the household. The tuberculin test is a screening, rather than a strictly diagnostic measure. The interpretation of a positive reaction in adults is dependent on the results of other diagnostic examinations; a positive reaction in children suggests a fairly recent infection. Modern roentgen-ray technics afford valuable records which aid clinical study. For large-scale investigations the use of photofluorographic units makes possible mass surveys. Clinical manifestations include evidence of loss of weight and appetite, rise in temperature late in the day, and physical evidence of the various manifestations of the disease.

Bacteriologic Examinations. Bacteriologic examinations and animal inoculation are the specific means of confirming a diagnosis. Since the bacilli may exist in small numbers in destructive lesions or may be liberated only intermittently, repeated and careful examinations must be made before excluding possible infection with

tubercle bacilli. The body secretions and fluids which are examined most frequently are sputum, gastric washings, urine, feces, and spinal, pleural, and ascitic fluids. Details for the collection and handling of these specimens will be found in appropriate chapters in Part IV.

Direct Microscopic Examination. Direct microscopic examination of carefully selected purulent or cheesy particles from sputum is recommended as a preliminary simple and time-saving procedure. Failure to find bacilli does not rule out the possibility of infection. More delicate methods and repeated trials are often required to ensure a correct diagnosis.

Concentration Methods. Concentration methods are often of value and a great many modifications of the original antiformin technic have been described. These methods depend upon the digestion of the mucus of the sputum and the subsequent concentration of the tubercle bacilli by centrifugation. In the process of digestion, the contaminating non-acid-fast bacteria are usually destroyed. The method of Hanks (1938) is given here. The minimal alum flocculation provides an excellent means of concentrating small numbers of tubercle bacilli, and is of especial value with pleural, spinal, and other body fluids.

A suitable volume (e.g., 5 ml.) of sputum, sediment from gastric washings, or other material, is mixed with an equal volume of 4 per cent sodium hydroxide; 0.2 per cent potassium alum and 0.004 per cent phenol red, or other suitable indicator, are also added. The whole is shaken and digested, with frequent agitation, in a water bath at 37° C. until homogenization is complete (usually about 30 minutes). (Andrus and McMahon (1924) digest their suspensions at 55° C.) When homogenization is complete, the entire digest is neutralized with approximately 2.5 ml. of a normal solution of hydrochloric acid (25 per cent HCl by volume). The acid should be added drop by drop and the protein precipitate dispersed by shaking. An overstepped end-point may be corrected by adding a drop or so of the 4 per cent sodium hydroxide digestor. A very fine flocculation at neutral point acts as the collecting agent for the tubercle bacilli. Centrifugation often requires only five minutes at high speed. The entire supernatant fluid is discarded and the sediment used for the preparation of slides and cultures or for the inoculation of guinea pigs. Very dense sputa require extra volumes of digestor. Failure to flocculate indicates the need of at least one additional volume of digestor, further shaking, and re-neutralization. With clear materials, such as spinal fluid, it is obvious that the digestor is not needed.

Chloroform concentration is also valuable. In this method only the sodium hydroxide is added to the sputum. After digestion and neutralization, chloroform equal to 2 per cent of the total volume is added, and the whole shaken vigorously to ensure emulsification. After centrifugation for five minutes the supernatant fluid is discarded and the creamy sediment filmed on slides.

Diagnostic Films. In the preparation of diagnostic films many of the bacilli may be lost if the usual routine of fixation is followed. After the films are spread, dried, and heat-fixed, they should be fixed further with methyl alcohol, or a protective coating of 0.5 per cent gelatin added, and the slides exposed to formalin vapor for five minutes.

Cultures. Cultures can be made directly upon suitable media from material which is not contaminated. Cultures from the sediments of pleural or other exudates, or the film from a tuberculous spinal fluid, may show tubercle bacilli when they are not demonstrable by smears or by guinea-pig inoculation. Cultures are also made from sputum or other contaminated material, for the methods of concentration used generally kill most of the non-acid-fast organisms present. Two or three drops of sediment should be spread evenly

over the surface of two to six tubes of the selected medium and the medium incubated with its surface horizontal until all fluid is absorbed. Then the tubes should be sealed tightly and stored in an erect position. Pure cultures can often be obtained in this way. Clough has grown tubercle bacilli from the blood in cases of miliary tuberculosis by laking the red cells with sterile distilled water, and planting the sediment after centrifugation on blood agar slants. The bacilli are rarely present in the blood in other forms of tuberculosis, however. Growth is slow and even under the most favorable conditions colonies do not become visible in less than a week. Within a few days, however, the tubercle bacilli will have multiplied sufficiently to demonstrate them in smears from the culture. The choice of a medium may be based on personal preference. The use of bacteriostatic agents will give fewer contaminants, but the omission of such agents in the media may yield more positive cultures. The egg-yolk medium of Dorset is still one of the most popular. Several other media are described in Chapter 11. Many workers favor the use of several kinds for each isolation.

Instead of using the sediments obtained in the concentration methods described above for cultures McNabb (1936) prefers treating the contaminated specimen with an equal volume of 3 per cent hydrochloric acid plus an indicator. After this mixture has stood for two hours it is made slightly alkaline with 3 per cent sodium hydroxide, centrifuged aseptically, and cultures made from the sediment.

Guinea-pig Inoculation. The guinea pig is susceptible to both bovine and human tuberculosis, and is used to demonstrate the organisms when they cannot be found by other methods, and to distinguish between tubercle bacilli and nonpathogenic acid-fast bacilli.

The animal is injected subcutaneously in the groin. If the material is badly contaminated, it must be treated with acid or alkali, as in making cultures, or the animal may die from some secondary infection. If the tubercle bacillus is present, a local swelling develops which may caseate and ulcerate. Smears from the ulcerated area often show the bacilli. The regional nodes become involved and later the mesenteric and other lymph nodes. The lesions may be looked for in from four to six weeks and death occurs in about two months or more. At autopsy the characteristic lesion is an enormous enlargement of the spleen, which is studded with grayish or yellowish tubercles. Smears and cultures from the spleen show the tubercle bacilli. The liver, lungs, and peritoneum are often involved. Block suggests that the lymph nodes in the groin be damaged by squeezing the tissue between the fingers before inoculating the animal and claims that tubercle bacilli may be demonstrated in these damaged nodes within 10 or 12 days. Infected guinea pigs become highly sensitive to tuberculin after two or three weeks, and will die acutely within 24 hours if given a subcutaneous injection of a large dose (1 or 2 ml.) of O.T. at this time. This phenomenon is utilized to determine whether or not tuberculosis has developed from the inoculation without waiting for the appearance of gross lesions. It is desirable to inoculate a second animal as a control and allow it to live until lesions have developed. Intracutaneous tuberculin tests may be used also to determine whether or not an inoculated animal has become infected.

Tuberculin. Tuberculous persons or animals become strikingly hypersensitive to subsequent injection of heat-killed bacilli (Koch phenomenon) and to tuberculoproteins in concentrations which produce no effect in nontuberculous individuals. This form of sensitivity differs from anaphylaxis or atopy in that it is apparently not mediated by a humoral antibody mechanism. The reactions are always delayed, and depend on a sensitivity of the tissue cells themselves.

Tuberculin hypersensitivity is the best known and the most typical of the bacterial allergies. The reactions are always of the "delayed" type, and should be read after an

interval of 48 to 72 hours. A positive reaction is indicated by definite inflammation and induration.

A positive reaction reveals some degree of past or present infection, but its significance in terms of active tuberculosis must be ascertained by careful roentgen-ray, clinical, and bacteriologic studies. Even in clinically active tuberculosis there is no constant relation between the degree of hypersensitivity and the severity of the disease. Reactivity may disappear in overwhelming tuberculous infection, or be temporarily suppressed by other acute infections, notably influenza, measles, and the exanthems.

The general incidence of positive tuberculin reactions in the population of the United States has now fallen below 50 per cent for persons of all ages. Although a positive tuberculin reaction still has greater clinical significance in children and in young adults below the age of 30, it is recognized as an important test in persons of all ages when tuberculosis is suspected, or is to be eliminated as a diagnostic possibility. Except in moribund individuals or in the presence of intercurrent infections, a negative reaction is of great significance.

The term "tuberculin" is applied to any of a number of protein derivatives from cultured bacilli. The reactions induced by these products may be classified as: (1) local inflammation or necrosis in ophthalmic reactions or skin reactions induced by patch tests, scarification (von Pirquet), or intradermal injection (Mantoux); (2) focal reactions of inflammation, hemorrhage or necrosis at the site of pre-existing lesions; e.g., in pulmonary tuberculosis there is increase in cough and sputum, and sometimes hemoptysis; in joint tuberculosis, increased pain, swelling, and disability of the joint; and (3) general reactions with fever, malaise, prostration, and even death. Obviously focal and general reactions are to be avoided in clinical work. The standard method of tuberculin testing today is by the intracutaneous injection of known dilutions of standardized tuberculin.

TUBERCULIN PREPARATIONS: KOCH'S "OLD TUBERCULIN" (O.T.). Formerly, Koch's "Old Tuberculin" was the standard tuberculin. It was made by heating a six- to eight-week glycerol broth culture at 80° C. until reduced to one-tenth of its original volume, and then filtering. In making dilutions for skin testing, 1 ml. of O.T. is regarded as 1000 mg. For survey purposes the initial test dose was usually 0.1 ml. of a 1 : 10,000 dilution containing 0.01 mg. Persons failing to react to this dose received a second injection of 0.1 ml. of a 1 : 100 dilution containing 1 mg. These two doses are suitable for the average subject, but it is wise to start with solutions at least 100 times more dilute when testing persons known to be suffering from inflammatory diseases of the eye or to have active tuberculosis and a high sensitivity.

PURIFIED PROTEIN DERIVATIVE (P.P.D.). The purified protein derivative of Seibert is a highly purified and carefully modified derivative of tuberculoprotein from cultures on Dorset's synthetic medium. It is the least antigenic of all tuberculins, free of constituents from the medium, and keeps indefinitely as a dry powder. It is now available in tablet form in two concentrations. The first dose of 0.00002 mg. of P.P.D. is contained in 0.1 ml. when one of the weaker tablets is dissolved in 1 ml. of phosphate buffer solution. Individuals who fail to respond to the first dose are given a second injection of 0.005 mg. (250 times the first dose), prepared by dissolving the stronger tablet as above. As with O.T., only one-hundredth of the weaker dose should be given to persons in whom marked sensitivity is suspected. This P.P.D. has largely replaced other tuberculins for diagnostic use.

Active Immunization. Although some degree of immunity can be produced in animals by the injection of dead tubercle bacilli, the protection so conferred is usually slight. On

the other hand, a guinea pig with an active tuberculous lesion containing living organisms of low virulence is more resistant to superinfection, even if a highly virulent strain is injected. Calmette has attempted to produce a similar resistance in man by feeding children with living cultures of an attenuated strain (B.C.G.) which, although producing tuberculin, has lost its pathogenicity by prolonged cultivation. The efficacy of this procedure can be judged only after prolonged trial. In this as in all vaccination with living organisms there is always a potential danger of a spontaneous restoration of virulence, and many investigators are opposed to this procedure on that account. Petroff and Steenken (1930) claim to have produced virulent, smooth forms from four different cultures of Calmette's B.C.G. On the other hand, many thousands of children have been treated in this way with practically no ill-effects which could be ascribed to the vaccine. The dangers involved in the immunization of children in homes with tuberculous adults are of little moment in comparison with the greater likelihood of the children acquiring the disease if they are given no protection at all.

Treatment with Antibiotics and Drugs. Streptomycin is the only antibiotic thus far reported to have therapeutic action in experimental tuberculosis. Tubercle bacilli may develop at least one thousand times their original resistance to this antibiotic. Streptomycin is less effective in pulmonary than in extrapulmonary tuberculosis. Final conclusions concerning its ultimate usefulness must await further study.

Various sulfone derivatives (diasone and promizole) have been reported as having some activity in pulmonary cases.

Leprosy

Mycobacterium Leprae (*Bacillus leprae*) (*Hansen, 1874*). This organism is accepted as the cause of human leprosy. It occurs sparsely in patients with high resistance and profusely in those who are highly susceptible. It is often very difficult to demonstrate in early cases before characteristic nodules have developed.

Morphology. In size, shape, and staining *M. leprae* is indistinguishable from the tubercle bacillus, but can be differentiated by the following points: (1) Leprosy bacilli are found ordinarily in huge numbers in the nodules (lepromas), chiefly within the so-called lepra cells, and are often grouped in packets, arranged in a palisade, or in masses called "globi." (2) Leprosy bacilli, both in the natural disease and experimentally, are very feebly antigenic, whereas tubercle bacilli are remarkably potent in eliciting reactions. (3) Leprosy bacilli do not resist the decolorization in acid-fast staining quite as well as the tubercle bacilli, although there is some variation in the individual strains in this respect. (4) Leprosy bacilli cannot be cultivated *in vitro* or transmit the disease to animals; guinea-pig inoculation provides a reliable differentiation from tubercle bacilli.

Cultivation. Cultivation of the leprosy bacillus has been attempted repeatedly and extensively, but with negative results. Hanks failed to observe multiplication of the bacilli in tissue biopsied from lepers and maintained as fibroblast cultures for periods of three to seven months *in vitro*. Some investigators have obtained rapid growths of acid-fast bacilli, both chromogenic and nonchromogenic. Reference has been made to these rapidly growing, acid-fast bacteria from leprous tissues in Table 7, on p. 77, and in the discussion on p. 76. Fraser and Fletcher, Eddy, Evans, and many others failed to obtain such cultures from lepers having no ulcerated lesions. Hanks cultivated them from nodules which were proximal to open ulcers, but never recovered them, over a period of six years, from the tissues in nonulcerated cases. All such strains that have been cultivated have been found by Gordon and Haagen in soils and have been classified by them as saprophytes. Cultivated diphtheroids have also been claimed as causative agents, but they are also associated with nodules exposed to secondary contaminations. All of these studies confirm the opinion of McCoy that the true leprosy bacillus has never been cultivated with certainty, and the

disease has never been transmitted artificially to animals or to man by direct inoculation of leprous tissue. Our knowledge of the behavior of the leprosy bacillus, therefore, is derived solely from observations of the disease.

Clinical Forms and Pathology of Leprosy. Leprosy differs from tuberculosis in several important respects: (1) It is not a destructive disease of the internal organs. Its manifestations are due largely to the presence of the bacilli in the connective tissue of the skin and the peripheral nerves. (2) It is not characterized by hypersensitivity. The cutaneous response to boiled bacilli corresponds to that of healthy individuals (in neural cases), or is absent (in lepromatous cases). (3) The causative agent has not yet been cultivated *in vitro*. A clear understanding of the bacteriologic aspects of the diagnosis of leprosy is dependent on recognition of the different clinical forms of the disease.

In *neural* (N) *leprosy* the infected persons show normal skin response to injected bacilli (lepromin), and the disease is confined to the peripheral nerves and to the corresponding leprid lesions in the skin in which bacilli are few or difficult to demonstrate. This state is associated with varying degrees of nerve enlargement, particularly of the ulnar, anterior tibial, peroneal, and other nerve trunks connected with the site of skin lesions. The skin manifestations include: (1) hypopigmented areas or macules, with a dry or crepe-paper appearance of the epidermis and loss of normal sweat and hair production, and (2) sensory disturbances which may include prickling sensations, inability to distinguish heat and cold, and relative degrees of anesthesia to light touch, deep pressure, or even to the pain of pin pricks or incision. Trophic bone absorption, contractures, deformities, and incapacities are common sequelae.

Sections from the involved nerve trunks or of the terminal skin lesions usually reveal multiple perivascular foci of round and epithelioid cells. The difficulty of finding bacilli in such lesions has been responsible for various theories concerning virus or non-acid-fast forms of the infectious agent.

A *tuberculoid subtype* of the neural form is characterized by skin lesions with distinctly raised and reddish margins. The margins may advance progressively over large and irregular areas like the spreading of a grass fire. Sections may reveal few or many bacilli. In this respect these lesions are intermediate between purely neural and lepromatous lesions. Their tendency to activation and subsidence has at times led to erroneously optimistic opinions concerning the efficacy of various forms of treatment.

Neural leprosy is less acute than the lepromatous form, and there is a higher incidence of spontaneous recovery. Patients with "burnt out" cases are paroled, but are kept under observation, since relapses are not uncommon.

In *lepromatous* (L) *leprosy* the skin becomes infiltrated with inconceivable numbers of bacilli which stimulate the formation of new tissue, making nodules. In such persons the tissue becomes indifferent to the presence of the bacilli, there is no response to the injection of killed bacilli, and susceptibility to the disease is at its maximum. The lepromas, or nodules, may appear in successive crops, associated with fever, malaise, and bacilli in the circulation, or they may develop insidiously as individual masses. They show a predilection for exposed or convex body surfaces, such as ear lobes, face, and testes. The membranes of the nasopharynx may become studded with nodules which, like those in the skin, may ulcerate and discharge enormous numbers of bacilli. Lymph nodes, spleen, and liver may accumulate large numbers of bacilli. The lungs are seldom involved.

The pathologic study of lepromatous lesions reveals a solid granulomatous infiltration with fibroblasts and macrophages, the latter being almost constantly stuffed with bacilli. These lesions develop in the corium, about the small vessels, sweat glands, and hair bulbs, and may replace the original structures; they also occur as freely movable nodules in the subcutaneous tissue. The stuffed macrophages constitute the so-called "lepra" or "foamy" cells in which the bacilli are often packed in bundles.

Mixed neural and lepromatous manifestations occur in many patients.

Epidemiology of Leprosy. Leprosy, though infectious, is but slightly contagious. There

have been but few cases among the staff personnel of leper colonies. Marriage between a leper and a nonleper on the average results in recognized infection of the spouse in only about 5 per cent of such marriages. Children are more susceptible than adults. Those in the Culion Leper Colony in the Philippines show their first lesions during the second year of life; in about 30 per cent of the children infection is manifest by the end of the third year. Some believe that infections may lie dormant from infancy until adolescence. In populations at large the majority of the cases are first recognized in individuals between 10 and 40 years of age. The disease occurs in all races and climates, but is most common today in China, India, Africa, the Philippines, Japan, and the Southwest Pacific.

Since the incubation period may be from 1 to 10 or more years, it is difficult to trace the transmission of the disease. In studies by Rodriguez and Doull near Cebu (Philippines) the incidence was about four times higher among those who had lived in households or in a small group of houses in contact with a known case than in those whose contacts were unknown. Since persons with L-type or bacteriologically positive cases disseminate enormous numbers of bacilli, the bacilli are doubtless inhaled and swallowed by close contacts and introduced into their cuts and abrasions. The first recognizable lesions tend to occur on exposed parts of the body in children, particularly on the buttocks, cheeks, legs, or arms, and not in the nasal mucosa.

All attempts to reproduce the disease by experimental inoculation of normal individuals have failed, but a few, unquestionable, accidental surgical infections have been reported. The apparently high resistance of human beings to experimental or contact infection is not yet explained.

Lepromin Reactions. As indicated, there is no increased hypersensitivity to leprosy bacilli or their extracts. A 5 per cent emulsion of boiled nodule in salt solution with 0.5 per cent phenol provides an abundance of bacilli for skin testing, and is known as *lepromin*. Following its intracutaneous injection, 95 to 98 per cent of normal persons and persons with neural cases respond with a slowly developing infiltration and induration during an interval of three or more weeks, which is known as a positive lepromin reaction. An almost correspondingly high proportion of lepromatous patients fail to react to human lepromin, but do react positively to lepromin prepared from the rat leprosy bacillus and to suspensions of acid-fast bacteria of the type which have been cultivated from leprous tissues. The relatively specific inability of lepromatous patients to respond to human lepromin and the positive response to other acid-fast bacteria afford an aid in differentiating true leprosy bacilli from related organisms.

The acute exacerbations, known as the lepra reaction, in the indolent course of the disease are believed to have an allergic basis, but involve minor degrees of elevated cell response which never approximate the sensitivity characteristic of tuberculous or other bacterial infections.

Laboratory Diagnosis. Laboratory diagnosis is secondary in importance to careful clinical observation and study, especially in very early or in neural cases. The demonstration of leprosy bacilli by staining is a very important step in the diagnosis of so-called bacteriologically positive leprosy, and the only laboratory diagnostic test of any significance. Owing to the desirability of ascertaining if cases can be classified as closed, or relatively noninfectious, it is a step of great importance from both the administrative and clinical point of view.

bacteriologic findings. In pure neural cases the diagnosis is usually dependent on clinical findings, and the prospects of demonstrating bacilli by any method short of biopsy and prolonged study of sections is rather poor. Owing to the possibility of mixed N and L infections, smears should be made from the nasal mucosa (after careful surface swabbing) by abrasion or scraping of the membranes themselves, and also scraped skin

incisions from the margins of macules, anesthetic areas, tuberculoid lesions, or suspicious blemishes. Success is dependent on sampling every site in which manifestations of leprosy are recognizable. Slides made from such sources must be searched carefully and patiently.

Because of the abundance and the widespread distribution of bacilli in lepromatous cases, their demonstration by scraped skin incision is very easy. The sites of first choice are the ear lobes, and any other skin areas which excite interest. It must be remembered that a case may be lepromatous without nodules and that the bacilli may be diffusely infiltrated throughout the skin. In a typical case all samples from skin lesions, nasal septa, lymph nodes, etc., will yield abundant bacilli, and no special search is required. Negative observations from lesions which appear lepromatous suggest that the case may actually be tuberculoid and that a very careful search for the bacilli should be made. Although leprosy bacilli have repeatedly been demonstrated in the blood of lepromatous patients, the results are too uncertain to be of diagnostic interest. In staining leprosy bacilli the Ziehl-Neelsen method is used.

It must be borne in mind that large numbers of acid-fast bacteria are required for their observation by microscopic methods, and that conclusions concerning the absence of bacilli are justified only after repeated sampling and study. This is especially true in patients who appear for primary diagnosis, or for prospective parole.

BLOOD AND SEROLOGY. In lepromatous leprosy the sedimentation rate of the red blood cells is increased in proportion to the gravity of the disease. These tests may provide indices of clinical progress, but are not diagnostic.

The Wassermann and the precipitation tests for syphilis give a falsely high incidence of positive reactions in leprosy, particularly in lepromatous cases. In general the incidence of positive reactions is associated with the gravity, or the reaction state, of the disease, and is not due to widespread yaws or syphilis in lepers. In the spinal fluid from lepers the serologic tests remain reliable.

CHEMOTHERAPY IN LEPROSY. The work with promine by Faget and others at Carville (1946) has been confirmed and extended by the use of promizole and diasone (1947), which have the advantage that they can be administered by mouth. These studies are of particular significance because they have been confined to lepromatous cases, the clinical type in which no treatment has hitherto been of benefit. Patients usually begin to improve only after six months of treatment. Results with promine, which has been studied over a period of four years, showed that the incidence of bacilli in the lesions had declined by the end of the second year and continued to decrease throughout the four-year period. Ulcers and secondary infections were controlled and there was no formation of new lesions. The drug appears to function as a bacteriostatic rather than a destructive agent for the bacilli. Though far from being ideal therapeutic agents, these sulfone drugs are the first which have been shown to be effective in lepromatous leprosy.

M. Leprae-murium (*Stefansky, 1903*). This bacillus is the cause of a natural disease in rats (rat leprosy) which is prevalent in Europe, Asia, and America. Morphologically it is indistinguishable from *M. leprae,* and attempts at cultivation have also been unsuccessful. In spite of their similarity the two organisms and diseases are distinct, as shown by the inability of the human bacillus to infect rats, and the frequently positive response of lepromatous individuals to rat lepromin.

The rat infection is always of the lepromatous type, and the lesions can develop in almost any part of the body. Histologic sections of the skin nodules show the same granulomatous infiltration in the corium and large rat lepra cells (histiocytes according to Oliver) packed with bacilli.

The disease can be transmitted to rats and some strains of mice. Infection takes place as readily through skin abrasions as by subcutaneous inoculation. It is believed that the natural infection is acquired through the skin, possibly from bites. Mechanical transmission by rat fleas is possible. Rat leprosy serves as an experimental disease in some phases of leprosy investigation.

Actinomyces and Nocardia

The actinomycetes, the causative organisms in actinomycosis, present characteristics of both fungi and bacteria and have been placed by some systematists in an intermediate position. For convenience, and to comply with recent bacteriologic usage, they are placed, together with the mycobacteria, in the family Actinomycetaceae.

Two genera are involved in actinomycosis infections: *Actinomyces* and *Nocardia.*

Characteristics. Members of both of these genera grow in delicate branching filaments about 0.5 to 0.8μ in diameter. These segmented filaments break up into fragments having the appearance of diphtheroids. They are Gram-positive and nonmotile. Some species of *Nocardia* are acid-fast. The genus *Nocardia* is aerobic and forms a mycelium which sends up short aerial hyphae giving a powdery appearance to the growth. The genus *Actinomyces* is anaerobic and does not form aerial hyphae, the growth being more or less rough, wrinkled, and waxy in appearance.

Although a number of species have been named, there are three members of this group of special medical importance.

Actinomyces Bovis. This species is the cause of actinomycosis, a condition which occurs most commonly as "lumpy jaw" in cattle and as localized cervicofacial or generalized infection in man.

This organism is anaerobic. It is found in the mouth, especially about the teeth and gums. Emmons (1936) found it in the crypts of 47 per cent of 100, and later (1938) in 37 per cent of 200, pairs of tonsils routinely examined. In man the infection is most commonly cervicofacial, but it may be thoracic or abdominal. Cervicofacial infection has the most favorable prognosis. *Actinomyces bovis* has never been isolated from soil or vegetation, and its natural habitat is probably the oral cavity. The disease is a chronic suppurative process characterized by formation of granulomatous lesions which break down and discharge through fistulae and sinuses. Bone may be also infected. The pus from the lesions commonly is found to contain small yellow granules ("sulfur granules," "Drüsen"), usually about 30 to 40μ in diameter, though they may be much larger.

These granules should be examined microscopically as fresh preparations by placing a loopful of material containing a granule on a slide and crushing it under a coverglass. If the sulfur granules are hard to find, Conant et al. (1945) suggest placing a piece of sterile gauze over a draining sinus overnight; the next morning granules may usually be found on the gauze. The granules will be seen, microscopically, to consist of one or more "rosettes." Each rosette is composed of a central core of tangled, branching filaments. The hyphae near the edge of the granule have a radial arrangement and terminate in club-shaped structures. This club appearance is caused by the deposition of material around the tips of the filaments.

Cultures are made by placing carefully selected and washed granules in chopped-meat broth, thioglycollate semisolid medium with dextrose, or deep dextrose veal infusion agar. The medium should be near neutral point. In liquid medium *A. bovis* grows in the bottom of the tubes as discrete, often "fuzzy," white colonies; in the veal infusion shake cultures the white cuneate colonies are found 5 to 10 mm. below the surface, and at greater depths. The cultures should be transferred every 10 days.

Nocardia Madurae. *Nocardia madurae* is one of the causes of mycetoma or Madura foot. It is probable that it grows as a saprophyte in the soil and enters the skin through punctures and abrasions. The organism is aerobic. Mycetoma occurs most frequently in tropical and subtropical zones, but may be found in many other regions where people habitually go barefooted. The infection may begin as a single papule or multiple papules which form abscesses and rupture with fistula formation. The whole foot (and occasionally hand) may become involved, including the bones. The condition is progressive and chronic, with no systemic reaction.

The diagnosis is made, as in actinomycosis, by examining granules microscopically and cultivating them. *N. madurae* produces white to yellow granules which are firmer than those of *A. bovis*. Other organisms may cause this clinical picture and produce granules of other colors.

Nocardia Asteroides. This microörganism is aerobic and acid-fast. Its habitat is soil, but it has been found in a number of lesions such as brain, lung, subcutaneous abscesses. It is blood-borne. It occasionally forms white granules but is usually found as scattered branching hyphae. *N. gypsoides* is probably identical with *N. asteroides*.

Actinobacillus Lignieresi. *Actinobacillus lignieresi* causes an infection in cattle which may be confused with cervicofacial actinomycosis. Only soft tissues are involved in this infection. Differential diagnosis can be made by microscopic examination and by culture. The organism has no relation to *Actinomyces* and is discussed on p. 122. Animal inoculation is of no value.

Corynebacteria

Diphtheria Bacilli and Diphtheroids

Corynebacteria are described in Bergey's Manual as slender, often slightly curved rods, with a tendency to clubbed and pointed forms, and with branching in old cultures. In staining they may appear solid, barred, or with granules. They are Gram-positive, non-acid-fast, nonmotile, and usually aerobic. The type species, *C. diphtheriae,* produces a powerful exotoxin, and is the cause of diphtheria. Twenty-one species of this genus have been named. The members of the group other than the diphtheria bacillus are usually termed "diphtheroid" bacilli. These diphtheroids are widely distributed in man, animals, and insects. They are relatively resistant to drying and may remain viable for a long time in air and dust. They are among the most common contaminants in bacterial cultures. Differentiation from the true diphtheria bacilli must often be made.

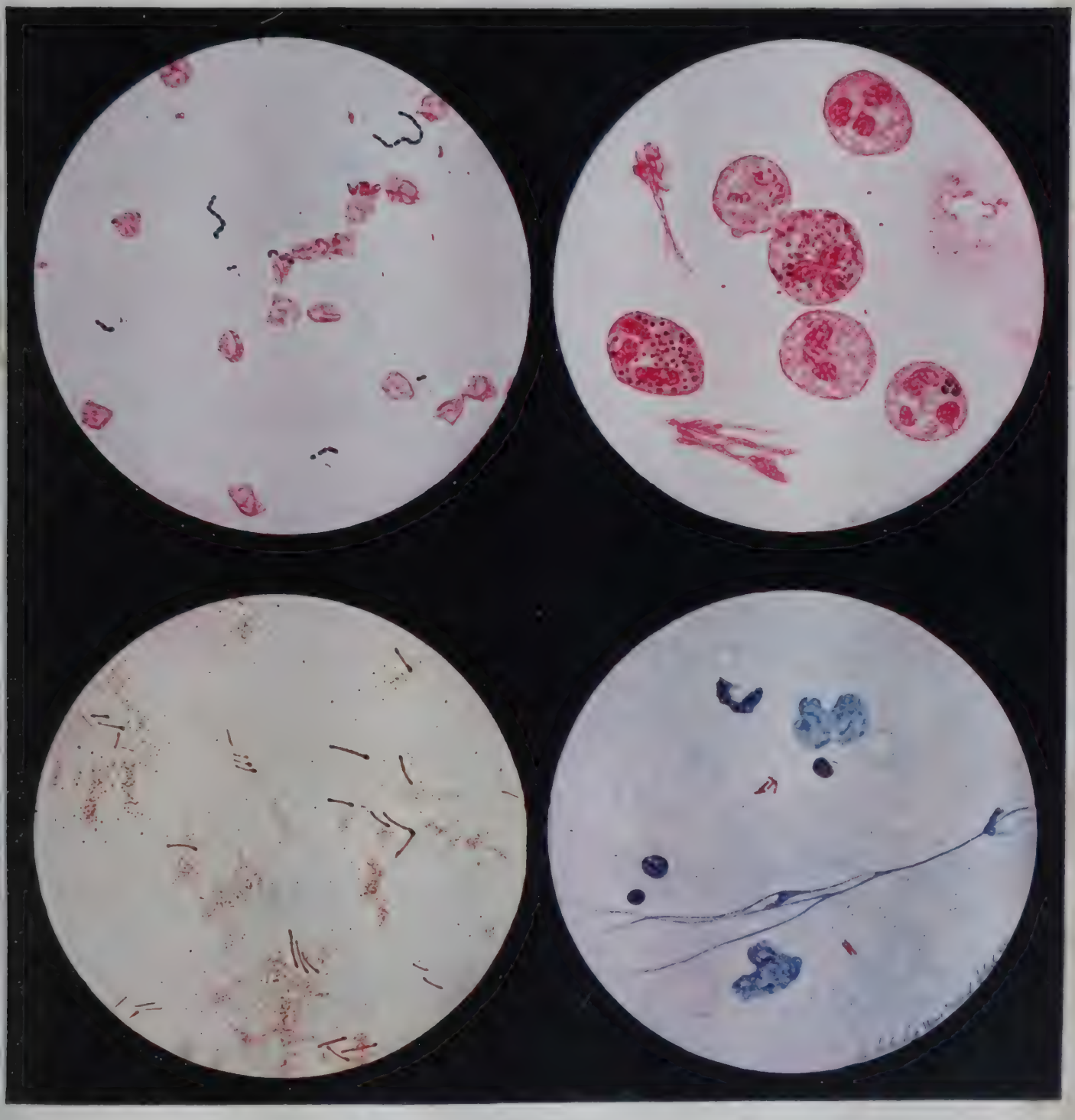


PLATE I

(*Upper left*) *Streptococcus pyogenes* (Gram's stain).
(*Upper right*) *Neisseria gonorrhoeae* (Gram's stain).
(*Lower left*) *Corynebacterium diphtheriae* (Neisser's stain).
(*Lower right*) *Mycobacterium tuberculosis* (Ziehl-Neelsen's stain).

Corynebacterium Diphtheriae (*Bacillus diphtheriae*) (*Discovered by Klebs, 1883, and Cultivated by Loeffler, 1884*). Diphtheria bacilli are present in large numbers in the characteristic ulcerations of the throat in diphtheria and in the secretions of the nose and pharynx. Infections of the nose, middle ear, and larynx occasionally occur. The mucous membrane of the vagina or the conjunctiva may be infected. Skin wounds may become infected. A form of tropical ulcer designated "Veld sore," common in various desert regions, has been shown to be due, in some instances, to the diphtheria bacillus. A diphtheritic angina may be complicated by a superadded infection with other organisms, particularly hemolytic streptococci or Vincent's organisms. In such cases the clinical picture may be misleading, and recognition of the underlying condition depends upon careful bacteriologic studies.

Morphology. The diphtheria bacillus is usually a slender, straight, or slightly curved rod with characteristic beading or banding, and small, deeply staining polar bodies or metachromatic granules at either end. The latter may be seen in an 18-hour culture, but are more abundant after 36 hours. The granules are brought out best with Neisser's stain, but Loeffler's methylene blue is the most satisfactory stain for general purposes. Some forms tend to be very short and to show a solid staining. In culture the bacilli may show pear-shaped or clubbed forms. They are frequently arranged in a V or Y figure, and, what is most characteristic, show a palisade arrangement. Short chains may occur. Diphtheria bacilli are Gram-positive, but not so tenacious of the gentian violet as are the cocci, and decolorization should not be carried too far.

Cultural Characteristics. The diphtheria bacillus grows best at 37° C. on media enriched with serum or blood. Loeffler's blood serum is the medium most commonly used for initial culture. The diphtheria bacillus grows so rapidly on this medium that it outstrips many of the contaminating bacteria. The colonies are at first small, whitish, and opaque; later the center becomes heaped up and the edges characteristically crenated. This organism grows luxuriantly on blood agar and, unlike the diphtheroids, often produces a narrow zone of hemolysis around the colonies. For isolation in pure culture, cystine tellurite blood agar is recommended (Frobisher, 1937). Potassium tellurite is reduced by the diphtheria bacillus, and the colonies, which are dark gray or black in the center with a lighter peripheral zone, stand out conspicuously. Many variations of potassium tellurite medium have been described. In broth the bacilli tend to grow on the surface and form a pellicle. Acid but not gas is produced in dextrose and levulose, and generally in galactose, maltose, dextrin, and glycerol. No fermentation occurs in lactose, saccharose, and mannitol. There is no proteolytic activity. Indol is not formed. Milk is not acidified or coagulated.

Variation. Smooth, rough, and intermediate types have been noted in cultures and also in the throats of convalescents. The S types lose their virulence in the transformation to the R forms. Typical, virulent S forms have not been produced experimentally from the R cultures. The wide divergence of morphologic forms is not considered as dissociation.

Types. Anderson, Happold, McLeod, and Thomson (1931) described three types of diphtheria bacilli which have since been observed by many other workers. The type designated as *gravis* was found associated with an especially severe clinical picture, and the type known as *mitis* with milder forms. The type *intermedius* lies between the two. Differences in morphology and cultural features have been described as characteristic of these types. On tellurite blood medium, colonies of the *gravis* type are irregular in outline and radially striated, with a very dark center, whereas *mitis* colonies are round, smooth, hemispherical, and slate-gray in color. The bacilli of the *gravis* type are inclined to be

short and solid-staining, to be nonhemolytic, and to ferment starch and glycogen. The *mitis* type usually shows granules, is hemolytic, does not ferment glycogen or starch. The *gravis* type has been found frequently in severe and intractable diphtheria in Europe, but has been infrequent in the United States. Frobisher (1942) questions the clinical value of type determination in individual cases since at least one week is required. However, such a procedure is undoubtedly useful in epidemics due to a special form, and gives valuable information to the epidemiologist.

Toxin and Toxoid. The diphtheria bacillus in its growth in the body and in suitable culture media produces a soluble toxin (Roux and Yersin, 1888) which is responsible for the systemic manifestations and sequelae of the disease. The toxins elaborated by different strains are antigenically similar, but the quantity produced varies greatly with the strain used, and with cultural conditions.

To prepare the toxin, broth cultures of a suitable strain, known to be a good toxin-producer, are made and incubated for about a week at 34° C. The medium is distributed in shallow layers in order to provide an abundance of oxygen which is needed for good toxin formation. A slightly alkaline reaction (pH = 7.8 to 8.0) and the absence of sugar are favorable. The culture is then filtered and stored in a dark, cool place to retard deterioration. After a preliminary diminution in toxicity known as ripening, the toxin remains relatively stable under suitable conditions. A single strain, isolated by Park and Williams (No. 8) in 1895, is still used extensively for commercial toxin production. An M.L.D. (minimum lethal dose) of toxin is the minimum quantity which will kill a 250-Gm. guinea pig in four days. The strength is usually expressed as a decimal of a ml.; e.g., 0.001 ml. = 1 M.L.D.

In the deterioration of toxin, substances are formed called toxoids, which, although not in themselves toxic, are capable of neutralizing antitoxin, and which are antigenic. This change can be accelerated by the addition of formalin (0.2 to 0.4 per cent). After several weeks incubation at 37° C. all of the toxin is converted into toxoid. The conversion is regarded as complete when 5 ml. injected subcutaneously or intraperitoneally into a guinea pig produces no effects. This toxoid is of great value in the active immunization to diphtheria.

Pathogenicity. Diphtheria bacillus infections, whether in the throat or elsewhere, are almost invariably localized, and the systemic manifestations are due to absorption of the soluble toxin into the blood stream. Although the disease does not occur naturally in animals, it may be produced experimentally in a number of species. Guinea pigs are very susceptible to the toxin and may be killed by as little as 0.00025 ml. Characteristic lesions are produced by the injection of the bacilli, which multiply locally and elaborate a toxin, or by the injection of the toxin itself. At the site of the inoculation there is an edematous, inflammatory swelling, with enlargement of the neighboring lymph nodes. The lungs are congested, and there are often hemorrhagic pleural and peritoneal effusions. The most characteristic finding in early deaths is in the enlarged and hemorrhagic adrenals. Frobisher (1942) has found that young chicks are susceptible to virulent diphtheria bacilli and to their toxin. He has used them successfully instead of guinea pigs for virulence tests.

Laboratory Diagnosis. This depends upon identification of the organism in

stained films from the exudate and from cultures made on Loeffler's blood serum. It is often necessary to demonstrate the pathogenicity of the strain by means of virulence tests in guinea pigs, rabbits, or chicks.

STAINED FILMS. On account of the distinctive morphology of the diphtheria bacilli it is possible to obtain immediate presumptive evidence in about 30 per cent of acute cases by demonstrating the organisms in smears from the local lesions. All antiseptic solutions should be discontinued several hours prior to obtaining the material for study. The smears should be stained with Loeffler's methylene blue or by Neisser's method. It is often helpful to make a Gram stain also, taking care not to carry the decolorization too far, since diphtheria and pseudodiphtheria bacilli, and the fusiform bacilli of Vincent are the only Gram-positive bacilli likely to be found in the throat. The latter stain readily with Loeffler's stain, and are conspicuous on account of their size and distinctive morphology. The spirochetes, with which they are always associated, are usually demonstrable with a good Loeffler stain although they may require a stronger one. Recognition of these organisms is very important since fusospirochetal infections may be indistinguishable clinically from diphtheria, or may complicate it.

CULTURES. Cultures on Loeffler's medium should be made in all cases. Growth on this medium is rapid, and smears from the growth will usually show the diphtheria bacilli within 12 to 18 hours. If negative the cultures should be re-examined after 24 and again after 48 hours. Cultures from the nose and throat are also used to determine the period of isolation and to detect carriers. Since avirulent organisms are frequently present in normal throats it is often necessary to test the virulence of the organism found. For this purpose a pure culture must be obtained by plating, preferably on a tellurite blood agar plate. Obtaining a pure culture, identifying it by fermentation tests, and determining its pathogenicity by virulence tests constitutes completed evidence for diagnosis.

VIRULENCE TEST: (*a*) *Subcutaneous Method*. Two ml. of a well-grown 48-hour broth culture are injected subcutaneously into a young guinea pig weighing from 200 to 250 Gm. If the organism is virulent, death will occur in from two to four days with the characteristic pathologic changes previously described. A control test should be made on a guinea pig which has previously received 500 units of diphtheria antitoxin.

VIRULENCE TEST: (*b*) *Intracutaneous Method*. Fraser and Weld (1926) described an intracutaneous method, using either rabbits or guinea pigs, which permits the use of the same animal for both "test" and "control" injections. A suspension of the culture to be tested is injected intracutaneously into a nonimmune animal; four to five hours later 500 to 1000 units of antitoxin are injected (intravenously into rabbit and intracardially into guinea pig). Immediately thereafter the original culture suspension is injected into fresh areas. Lesions reach their height in the guinea pig in 48 hours and in the rabbit in 72 hours. A positive reaction is manifest by a central necrotic area surrounded by a zone of redness; the negative reaction is indicated by a small, pinkish papule. "Field cultures" may cause complications in reactions, so should be used only when necessary; in such cases the guinea pig is the better animal.

Several strains may be tested on one animal by giving 0.15 ml. of the suspended culture of each intradermally, and observing the local lesion. A control pig which has been injected 24 hours previously with 500 units of antitoxin is inoculated in the same way. If the culture is virulent the test pig will develop an inflammatory reaction and later an area of necrosis about the puncture, while the control pig shows no reaction. Avirulent organisms produce no reaction in either animal.

VIRULENCE TEST: (*c*) *Frobisher's (1942) Chick Method*. Seven- to twenty-day-old chicks are used. About 2 ml. of a 48-hour broth culture is injected into the subcutaneous areolar tissues dorsally between wing insertions. Only pure cultures are used. About one hour

before injection, control chicks are given 50 to 100 units of antitoxin in the same area that is to receive the culture. Virulent strains will cause a typical wing paralysis to develop in the chicks and will kill them in 24 to 72 hours. Nonvirulent strains and diphtheroids do not affect the chicks.

ANTITOXIN UNIT. The toxin produced by the diphtheria bacillus, when injected into animals, stimulates the production of a neutralizing antitoxin. The potency of an antitoxic serum is expressed in terms of antitoxin units. The unit, as originally defined by Behring, was the quantity which would protect a 250-Gm. guinea pig from 100 M.L.D. of a standard toxin. Since the toxin is unstable, it is less suitable for a permanent standard than is the antitoxin which can be preserved for long periods without deterioration. Antitoxin is now universally used as the permanent standard. In the United States the permanent standard is an antitoxin prepared by the National Institute of Health at Washington. The unit of this serum has been adopted arbitrarily and bears no relation to the original antitoxin unit of Behring and Ehrlich or to any "standard" toxin. Samples can be obtained by manufacturers by means of which they can determine the strength of their own (ripened) toxin preparations, and from this the potency of new antitoxic sera.

STANDARDIZATION OF TOXIN AND ANTITOXIN. The strength of the toxin is measured by mixing various amounts with one unit of standard antitoxin, and determining the minimum amount of toxin which, in such a mixture, will kill a 250-Gm. guinea pig in four days. This quantity of toxin is termed the L_+ dose. The protocol as shown below illustrates such a test. The L_+ dose of this toxin is 0.230 ml.

To standardize a new antitoxin, this L_+ dose of toxin is then mixed with varying quantities of the new antitoxin, until a mixture is obtained which will kill a 250-Gm. guinea pig in four days. This amount of antitoxin contains one unit, and the number of units per ml. can be calculated. The following protocol illustrates the titration of a

Dose of Toxin, Milliliters	*Dose of Antitoxin*	*Death*	*Lesions*
0.225	1 unit	5 da. 6 hr.	Characteristic
0.230	1 unit	4 da. 4 hr.	Characteristic
0.235	1 unit	3 da. 9 hr.	Characteristic

new antitoxin with the L_+ dose (0.23 ml.) of this toxin. In this test 0.0125 ml. of the new antitoxin protects for three days and 22 hours, and, therefore, contains one unit, or, by calculation, 80 units per ml.

Dose of Toxin, Milliliters	*Dose of Antitoxin*	*Death*	*Lesions*
	(Control standard)		
0.23	1 unit	4 da. 4 hr.	Characteristic
	(Serum unknown)		
0.23	0.01 ml.	2 da. 10 hr.	Characteristic
0.23	0.0125 ml.	3 da. 22 hr.	Characteristic
0.23	0.015 ml.	7 da. 6 hr.	Characteristic

The L_+ dose has no constant relation to the M.L.D. Precise determinations of the latter are more difficult, and are unnecessary for standardizing antitoxin. The amount of toxin which is exactly neutralized by one unit of antitoxin is termed the L_0 dose. Theoretically, the difference between the L_0 dose and the L_+ dose should equal 1 M.L.D. Actually, however, this difference is considerably greater because toxin preparations contain other

substances in large, but varying amounts, which neutralize antitoxin but do not produce toxic effects in animals. In a neutral mixture, a significant part of the antitoxin may be bound by these substances.

A reasonably accurate economical method of standardization is to inject the toxin-antitoxin mixtures intracutaneously into guinea pigs and note the presence or absence of a local inflammatory reaction (Schick reaction).

An approximate preliminary standardization can be made by Ramon's flocculation test which depends upon the fact that in a series of toxin-antitoxin mixtures, flocculation occurs first in that mixture which is most nearly neutralized. Although this flocculation test shows neutralization of a certain quantity of antitoxin, it does not necessarily indicate the unit value found by the guinea-pig method.

Schick Test. Most normal adults are immune to diphtheria owing to the presence of natural antitoxin in their blood. If a small amount of the toxin (1/50 M.L.D.) is injected intracutaneously into immune individuals, it will be neutralized by the natural antitoxin, and no reaction will occur. If no natural antitoxin is present, there will result a local inflammatory reaction. This is known as the Schick test, and is of great value in the determination of susceptibility and the effectiveness of immunization. Clinical observations have shown that the quantity of circulating antitoxin required to give a negative reaction protects an individual from the disease, even when virulent organisms are harbored in the throat.

The Schick test is performed as follows: Into the forearm, 0.1 ml. of diphtheria toxin, representing 1/50 M.L.D., is injected intradermally. A positive reaction shows within 48 hours, and reaches its maximum intensity in five to seven days as a reddened area 1 to 3 or more cm. in diameter with more or less induration. The intensity of the reaction is

Table 8

Average Susceptibility of Various Ages to Diphtheria (as Indicated by the Positive Schick Diphtheria-toxin Skin Test) in New York City (after Park)

Age	*Schick + (Susceptible) Per Cent*
Under 3 months	15
3 to 6 months	30
6 months to 1 year	60
1 to 2 years	60
2 to 3 years	60
3 to 5 years	40
5 to 10 years	35
10 to 20 years	25
20 to 40 years	18
Over 40 years	12

inversely proportional to the amount of natural antitoxin present. The reaction persists for about a week leaving a brownish pigmentation. It is important to inject an equal amount of the diluted toxin heated to 75° C. for one hour into the other arm as a control. A reaction occurring with the heated toxin is known as a pseudoreaction, and is regarded as an allergic response to certain constituents in the broth culture. Certain types of peptone are

especially apt to cause such a reaction. It has nothing to do with susceptibility to diphtheria toxin since this is destroyed by the heating. It appears sooner than the true reaction, is more urticarial in type, and disappears more rapidly, usually within 48 hours. Combined reactions may occur and require considerable experience for their correct interpretation.

The great value of the Schick test is in the determination of susceptibility. Table 8 shows the percentage of susceptibles in different age groups. The low percentage of positive reactions in subjects under six months of age is due to a transient immunity inherited from the mother.

Serum Therapy in Diphtheria. In the preparation of antitoxin, horses are injected subcutaneously with the toxin or broth filtrate at weekly intervals for three or four months. When each ml. of the serum is found to contain about 250 to 500 antitoxin units the horse is bled from the jugular vein. Some sera contain as much as 1300 units in 1 ml. Methods of purifying and concentrating antitoxin are now generally employed which depend upon the principle that the antitoxin in the horse serum is precipitated with the globulins which come down when various precipitating agents are used. By thus eliminating other proteins, the total amount of foreign protein per unit is reduced and the dangers of a serum reaction correspondingly lessened.

Antitoxin should be given promptly as soon as the diagnosis of diphtheria is made. If the case is clinically suspicious, antitoxin should be administered without waiting for the result of a culture. The statistics of Zingher indicate that the mortality approximately doubles with each day that the administration is delayed. In mild cases 10,000 units are given. In the malignant cases as much as 40,000 units should be used. The antitoxin is usually given by intramuscular injection (not subcutaneously), but in severe cases 5000 to 10,000 units should also be given intravenously, particularly if the larynx is affected, or if administration has been delayed. It is desirable that the entire amount needed be given on the first day. Much larger doses are sometimes given, but it is probable, as Park believed, that nothing is gained by exceeding these quantities, or by repeating these doses daily. Tests for hypersensitiveness (see p. 295) should precede administration.

Immunization: passive immunization. Passive immunization with 500 units of diphtheria antitoxin is given to Schick-positive contacts when it is necessary to produce immunity quickly. This, however, protects only for two or three weeks.

active immunization. When diphtheria toxin is treated with certain substances such as formalin, its toxic properties are destroyed by conversion into toxoid, while its immunizing ability is retained. This product has been used extensively in active immunization. Three injections are given. It may, however, cause marked general reactions in older children and adults, owing to a hypersensitiveness to the proteins in the broth culture. These individuals give a pseudoreaction to the heated toxin used as a control in the Schick test. When this occurs the initial dose of the formol toxoid should be small, the quantity being increased if the reactions are not severe. If this reaction is very marked toxoid-antitoxin floccules (purified precipitate formed by the action of antitoxin on toxoid) can be used.

The precipitation of toxoid by 2 per cent alum (Glenny, 1931) increases its efficacy. Two injections of this preparation are given about six weeks apart. Both toxoids are carefully standardized and the dosage is indicated on the labels.

The immunity produced by these methods develops within a few months and may persist for years. Maintenance of the immunity, however, appears to depend upon casual contacts with diphtheria-bacillus carriers. The development of immunity is determined by the Schick test. For the complete control of the disease all children of preschool age with a positive Schick reaction should be immunized. The effective use of the antitoxin in treatment and particularly the immunization of susceptible children have reduced the mortality from diphtheria in New York per 100,000 from 86 in 1895 to 1899 to 2.6 in 1931 and 0.2 in 1946.

Diphtheria Carriers. Diphtheria is spread by convalescent and healthy carriers, and their detection is, therefore, of great importance. The organisms may occur either in the throat or nose, and both must be examined. Ordinarily a convalescent becomes free from the bacilli within a few weeks, but occasionally a chronic carrier state develops. The bacilli may lodge in the crypts of the tonsils, and tonsillectomy may be necessary to clear up the condition. It must be remembered, however, that some morphologically typical bacilli are not virulent, and that quarantine of persons showing only avirulent organisms is not justified. The pseudodiphtheria bacillus is a common inhabitant of the throat, but it can usually be distinguished morphologically from the true diphtheria bacillus. If any doubt arises the virulence must be tested.

Corynebacterium Pseudodiphtheriticum (*B. hoffmannii*). This bacillus is a harmless saprophyte which is frequently found in healthy throats. It has no medical importance except that it must be distinguished from the diphtheria bacillus. This is usually possible by the morphologic and cultural characteristics alone and always by guinea-pig or chick inoculation.

Pseudodiphtheria bacilli usually tend to stain solidly, or to have at most only a single unstained segment. They are shorter, thicker, stockier, and do not curve so gracefully. They grow more luxuriantly, and are often chromogenic. There is no hemolysis on blood agar. They produce very little acid on sugar media; some produce none. They do not produce a toxin, and are not pathogenic for guinea pigs.

Corynebacterium Xerose (*B. xerosis*). This organism was first isolated from the conjunctiva of a form of chronic conjunctivitis known as xerosis, but since it is frequently present in the normal conjunctival secretion its pathogenicity is dubious. It resembles the diphtheria bacillus closely but differs in the following respects: (1) It does not usually show polar bodies with Neisser's stain. (2) It grows more slowly and delicately. (3) It forms acid from saccharose and dextrose but not from dextrin. (4) It is not pathogenic for guinea pigs.

The following table gives the differentiation of three of the corynebacteria by their fermentation reactions.

Table 9

Fermentation Reactions of Corynebacteria

Species	*Dextrose*	*Saccharose*	*Dextrin*
C. diphtheriae	+	—	+
C. xerose	+	+	—
C. pseudodiphtheriticum	—	—	—

Other diphtheroid bacilli have been isolated from many different sources—skin, urine, ascitic fluid, spleen, lymph nodes, etc. Their presence in the lymph nodes has led to the belief that they were the etiologic agent in various diseases—Hodgkin's disease, arthritis, leprosy, etc. They are found so frequently in normal tissues, however, that the assumption that they are the cause of any specific disease from this finding alone is unwarranted. They are among the most insidious and troublesome contaminants in cultures from human tissues.

Listeria and Erysipelothrix

Listeria Monocytogenes (*Murray, Webb, and Swann, 1926*). *Listeria monocytogenes* is the only species in its genus. It is a very small Gram-positive rod which is motile. In morphology, it bears some resemblance to small diphtheroids and is found singly, in V and packet arrangements, as well as sometimes in short chains. It is cultivated readily upon enriched media, and on blood agar the round transparent colonies are hemolytic. It is aerobic and facultatively anaerobic. It is not very active biochemically, though it produces acid in dextrose, rhamnose, and salicin. It does not liquefy gelatin, form indol, nor reduce nitrates. Julianelle (1939) has described two serologic types.

This organism is pathogenic for a number of animals. It produces an encephalitis in ruminants and in swine, and a generalized infection in fowls and rodents, especially mice. There is a marked increase in mononuclear leukocytes in these infections. Listerellosis also occurs in man, and the organism has been isolated from at least 20 cases. It is suspected to be the cause of infectious mononucleosis in man, but its etiologic role in this disease is not definitely proved.

Erysipelothrix Rhusiopathiae. This organism is important chiefly as the cause of swine erysipelas. It may also produce a disease in man termed "erysipeloid," as distinguished from true erysipelas of streptococcal origin. It is contracted by man through skin abrasions in handling materials of animal origin, or fish and shellfish. In the United States it is often called "fish-handlers' disease." Although human infection may occasionally be septicemic, the usual type is an edematous erythema occurring on the hands, which runs a self-limited course of about a month.

The organism is a small, slender, nonmotile, Gram-positive rod, which grows well on ordinary culture media, and is somewhat micro-aerophilic. Smooth colonies are very small, round, and transparent; the rough form occurs in long chains, and forms colonies resembling anthrax although very much smaller. Rough cultures grow in broth in masses of tangled threads. Fermentation reactions are variable; indol is not formed, but nitrates are reduced and hydrogen sulfide is produced. Diagnosis is by cultivation of the organism from the lesion.

Lactobacilli

Lactobacilli comprise a group of non-spore-bearing, nonmotile bacilli, varying in size from small coccobacillus types to long slender rods. These bacilli are Gram-positive but are relatively easily decolorized; even young cultures may contain some cells that appear Gram-negative, and old cultures may seem entirely so. Sometimes the organisms have a granular or beaded appearance. They may occur singly, in chains or filaments, or in palisades. Surface colonies are usually 1 to 1.5 mm. in diameter, and may appear smooth or rough. Lactobacilli produce acid (lactic), but not gas, from many carbohydrates, and are capable of resisting a considerable concentration of acid in their environment, thereby being sometimes called the "aciduric" group of bacteria. They do not grow in carbo-

hydrate-free media, and also require vitamin B fractions such as liver, yeast extracts, or tomato juice. Most species grow either under aerobic or anaerobic conditions, but anaerobiosis is often preferred.

Some species are normal inhabitants of the mouth and intestinal tract, constituting the major portion of the intestinal flora of nursing infants. They are also found in the vagina and in milk and cheese.

Classification of the lactobacilli is not entirely satisfactory. Many varieties, named according to source, have been found to be identical with strains from other sources. At least 15 species seem to be well recognized, though about 40 have been described.

None of these microörganisms are known to be pathogenic; however, *L. acidophilus* has been shown to be closely associated with lesions of dental caries. By the use of lactobacillus counts in saliva it is possible to classify individuals as to whether or not they are susceptible to dental caries; and also to determine whether or not the disease is in an active stage in the individual.

Within the past 10 years, some of the lactobacilli have been utilized in the biologic assay of both vitamins and amino acids. (Synthetic media with a casein base are generally used for this purpose (Snell, 1945)).

Lactobacillus Acidophilus (*Moro, 1900*). This bacillus is a normal inhabitant of the mouth and intestines of more than 90 per cent of individuals. A high count in the saliva is usually associated with a high count in the intestines, and it has been suggested that those found in the intestines have been swallowed. The Boas-Oppler bacillus (*L. boas-oppleri*), reported as being recovered from stomach contents, is probably a variety of *L. acidophilus*. *L. bifidus* (Tissier, 1900), so named on account of the frequency with which Y-shaped forms are found, occurs in the stools of nursing infants. It is usually described as an anaerobe, though after isolation it may become adapted to aerobic conditions. It is probably a variety of *L. acidophilus*. A carbohydrate environment favors the increase of *L. acidophilus* both in the mouth and in the intestines.

As mentioned above, a high incidence of *L. acidophilus* in the mouth is associated with dental caries. Lactobacillus counts are made by culturing saliva quantitatively on tomato-juice agar plates (Kulp and White, 1932) (see p. 357).

Döderlein's bacillus (1892), a common organism in the vagina, is identical with *L. acidophilus*. The marked acid reaction produced in vaginal secretions is thought to help prevent the occurrence of puerperal infection.

Cultures of *L. acidophilus* are used in the preparation of buttermilk.

Lactobacillus Bulgaricus. *L. bulgaricus* is very similar to *L. acidophilus* but is usually found as a saprophyte in milk and milk products. It was originally isolated from Yoghurt by Grigoroff (1905). Metchnikoff advocated drinking milk fermented with *L. bulgaricus* on the theory that this organism replaced putrefactive microörganisms in the intestines. Later experience has shown that *L. bulgaricus* is rarely implanted in the intestines, whereas *L. acidophilus* is easily established in such an environment. Cultures of *L. acidophilus* are now used when such fermented milk is desired.

Lactobacillus Casei, L. Arabinosus, and L. Fermenti. *Lactobacillus casei* has been used extensively in the assay of various vitamin B fractions, employing a synthetic medium of known composition, usually with a casein hydrolysate base. *L. arabinosus* and *L. fermenti* have also been used for this purpose and for the quantitative determination of certain amino acids. This subject has been thoroughly reviewed by Snell (1945).

CHAPTER 5

Gram-negative Bacilli

Key and Notes

Key to the recognition of non-spore-bearing, Gram-negative bacilli:

Family XI. Parvobacteriaceae. Small Gram-negative rods. Not active in fermentation of carbohydrates. Parasitic.

Tribe: Hemophileae. Minute rods, sometimes thread-forming and pleomorphic. Dependent on some factor contained in blood or plant tissues.

Aerobic.

Genus: *Hemophilus*. Nonmotile.

A. Chiefly affecting respiratory mucosa and conjunctiva.

1. *H. influenzae* (Pfeiffer's bacillus).
2. *H. suis* (associated with swine influenza).
3. *H. hemolyticus*.
4. *H. parainfluenzae*.
5. *H. pertussis*.
6. *H. conjunctivitidis* (Koch-Weeks bacillus). Regarded by many as identical with *H. influenzae*.

B. Affecting the genital region.

7. *H. ducreyi* (Ducrey's bacillus).

Genus: *Moraxella*.

1. *M. lacunatus* (*H. lacunatus, H. duplex,* Morax-Axenfeld bacillus).

Genus: *Noguchia*. Motile, encapsulated.

1. *N. granulosis*. Found by Noguchi in cases of trachoma.

Anaerobic.

Genus: *Dialister*. Nonmotile.

1. *D. pneumosintes* (*Bact. pneumosintes* of Olitsky and Gates). Found in nasopharyngeal secretions.

Tribe: Brucelleae. Small Gram-negative rods which grow on ordinary media.

Genus: *Brucella*.

1. *Br. melitensis* (*Bact. melitense, Mic. melitensis*). Cause of Malta fever.
2. *Br. abortus* (Bang's bacillus). Causes contagious abortion in cattle, brucellosis in man.
3. *Br. suis*. Causes contagious abortion in cattle and swine; brucellosis in man.
4. *Br. bronchiseptica*. Causes acute infection, chiefly respiratory, in dogs, cats, rabbits, guinea pigs, and other animals. Related to *Br. abortus*.

Tribe: Pasteurelleae. Small Gram-negative rods, showing bipolar staining. Aerobic.

Genus: *Pasteurella*.

Growth on ordinary media.

I. Nonmotile.

A. Indol and H_2S produced. No growth in bile. Sorbitol fermented. Many animal strains.

1. *P. avicida* (*P. aviseptica*). Causes chicken cholera. } *P. multocida*
2. *P. muricida* (*P. muriseptica*). Rodents, chiefly rats. }

3. *P. suilla* (*P. suiseptica*). Swine plague.
4. *P. cuniculicida* (*P. lepiseptica*). Snuffles in rabbits. } *P. multocida*

B. No indol nor H_2S produced. Growth in bile. Sorbitol not fermented.

5. *P. pestis* (*B. pestis*). Cause of plague.

II. Motile. H_2S produced. Indol not produced.

6. *P. pseudotuberculosis* (*Corynebacterium pseudotuberculosis*). No growth on ordinary media.

7. *P. tularensis* (*B. tularense*). Cause of tularemia. Requires special growth factors found in egg or blood; cystine.

Genus: *Malleomyces*. Short Gram-negative rods with rounded ends, sometimes in threads and showing a tendency to branch. Grow well in media containing body fluids. Highly parasitic.

1. *M. mallei* (*B. mallei, Actinobacillus mallei*). The cause of glanders. Nonmotile. No carbohydrates fermented.
2. *M. pseudomallei* (*B. whitmori*). Causes an infection resembling glanders. Motile. Some carbohydrates fermented. Gelatin liquefied.

Genus: *Actinobacillus*. Small Gram-negative rods, often coccoid, but may form chains in liquid media. Form granular aggregates in cultures and tissues.

1. *A. lignieresi*. Causes a disease resembling actinomycosis in cattle, goats, and sheep.

Tribe: Bacteroideae.

Genus: *Bacteroides*. Anaerobic.

1. *B. fragilis*.
2. *B. melanogenicus*.
3. *B. funduliformis*.
4. *B. serpens*.

Genus: *Fusobacterium*.

1. *F. plautivincenti*.

Family X. Enterobacteriaceae. Gram-negative rods.

Tribe: Escherichieae. Ferment lactose and dextrose with formation of acid and gas. Usually do not liquefy gelatin.

Genus: *Escherichia*. Methyl-red positive. Voges-Proskauer negative. Motile.

1. *E. coli*. Normal inhabitant of intestine of vertebrates. Widely distributed in nature.

Genus: *Aerobacter*. Methyl-red negative. Voges-Proskauer positive. Motile or nonmotile.

1. *A. aerogenes*. Widely distributed in nature.
2. *A. cloacae*. Liquefies gelatin slowly.

Genus: *Klebsiella*. Encapsulated. Nonmotile. Found principally in respiratory tract of man.

1. *K. pneumoniae* (Friedländer's bacillus, *B. mucosus capsulatus*).

Tribe: Salmonelleae. Motile and nonmotile; ferment numerous carbohydrates. Voges-Proskauer negative. Gelatin usually not liquefied.

Genus: *Salmonella*. Ferments dextrose with acid and gas.

1. *S. paratyphi A* (*S. paratyphi, B. paratyphosus A*).
2. *S. paratyphi B* (*S. schottmuelleri, B. paratyphosus B*).
3. *S. paratyphi C* (*S. hirschfeldii, B. paratyphosus C*).
4. *S. choleraesuis* (*B. suipestifer*).
5. *S. enteritidis* (*B. enteritidis*).
6. *S. typhimurium* (*B. aertrycke*).
7. *S. typhi* (*B. typhosus*, typhoid bacillus, *Eberthella typhosa*, *S. typhosa*). Motile. Acid without gas in many carbohydrates. Voges-Proskauer negative.

Genus: *Shigella*. Nonmotile. Acid without gas in many carbohydrates. Voges-Proskauer negative.

No acid from mannitol.

I. No indol.

1. *S. dysenteriae* (*B. dysenteriae*, Shiga, Shiga's bacillus).

II. Indol.

2. *S. ambigua* (Schmitz's bacillus).

Acid from mannitol.

I. No acid from lactose.

A. No acid from xylose.

3. *S. paradysenteriae* (*B. dysenteriae*, Flexner).

B. Acid from xylose.

4. *S. alkalescens*.

II. Acid slowly formed from lactose.

A. Indol formed.

5. *S. madampensis* (*S. dispar*).

6. *S. ceylonensis* (*S. dispar*).

B. Indol not formed.

7. *S. sonnei* (*B. dysenteriae*, Sonne).

Tribe: Proteae. Motile. Ferment dextrose, but not lactose, with acid and gas.

Genus: *Proteus*.

A. Liquefies gelatin. Acid and gas from mannitol and sucrose.

1. *P. vulgaris* (see footnote).

B. Does not liquefy gelatin. No acid and gas from mannitol or sucrose.

2. *P. morgani*.

Tribe: Serrateae. Small aerobic motile Gram-negative rods, proteolytic, nitrate-reducing, and usually producing a characteristic red pigment.

Genus: *Serratia*.

1. *S. marcescens* (*B. prodigiosus*).

Family IX. Achromobacteriaceae.

Genus: *Alkaligenes*. No acid produced from carbohydrates. Voges-Proskauer negative.

A. Motile. No gelatin-liquefaction. Found in intestinal tract.

1. *A. faecalis*.

Family II. Pseudomonadaceae.

Tribe: Spirilleae. Cells more or less spirally curved.

Genus: *Vibrio*. Short, bent rods, occurring singly or united into spirals. Motile.

1. *V. comma* (*V. cholerae, Spirillum cholerae asiaticae*). Cause of cholera in man.

2. *V. metchnikovi*. Pathogenic for fowls.

Tribe: Pseudomonadeae.

Genus: *Pseudomonas*. Produces a water-soluble, bluish- or yellowish-green pigment.

1. *P. aeruginosa* (*B. pyocyaneus*).

Family IV. Rhizobiaceae. Utilize carbohydrates without acid-production.

Genus: *Chromobacterium*. Produces a violet pigment soluble in alcohol.

1. *C. violaceum* (*B. violaceus*). Does not grow at 37° C.

2. *C. ianthinum*. Grows at 37° C.

OTHER GRAM-NEGATIVE MICROÖRGANISMS OF UNCERTAIN CLASSIFICATION:

1. *Streptobacillus moniliformis*.

2. Pleuropneumonia group of organisms.

3. Donovan's Bodies.

NOTE. Proteus X_2 and X_{19} used in Weil-Felix reaction are strains of *P. vulgaris* isolated from urine of patients suffering from typhus fever.

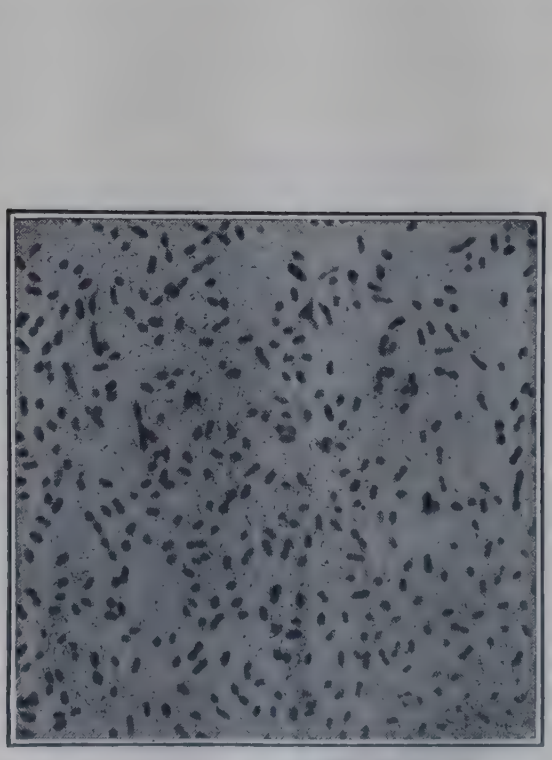
Gram-negative Bacilli Requiring Special Media

Hemophilus Influenzae (*Bacillus influenzae*) (*Pfeiffer, 1892*). This is the type species of the so-called hemophilic bacteria, most of which grow only on media containing special food factors present in red blood cells.

Morphology. *H. influenzae* is a small bacillus ($0.3 \times 1.5\mu$), nonmotile, and Gram-negative. Type-specific strains are encapsulated. In cultures it frequently shows involution forms, coccobacillary or filamentous forms. The latter have been noted particularly in strains isolated from the meninges. These organisms stain rather faintly, sometimes more deeply at either end so that they resemble diplococci. They may be well demonstrated by staining for five minutes with a 1 : 10 dilution of carbolfuchsin, or with Loeffler's methylene blue.

Culture. The influenza bacillus can be isolated on plates of rabbit's blood agar (human blood is often inhibitory), or on Avery's sodium oleate hemoglobin agar which inhibits Gram-positive organisms and favors the growth of the influenza bacillus. A good growth-promoting medium to which suitable dilutions of tyrothricin (Schoenbach, 1942) or penicillin (Fleming, 1929) are added facilitates isolation. Chocolate agar or broth, Levinthal's agar and broth (filtered chocolate medium), and Fildes' peptic digest of blood in agar or broth are particularly favorable. On blood agar the colonies are very minute, transparent and dewdrop-like, and are barely visible without a lens during the first 24 hours. They are invisible by transmitted light. On media more favorable than blood agar the colonies are much larger. On transparent medium those of type-specific cultures are mucoid, moderately opaque, and iridescent in transmitted light. Colonies of non-type-specific cultures are translucent and have a bluish sheen.

Two growth-promoting constituents must be present in the media to obtain a growth of the influenza bacillus. One factor, designated X, is an iron compound derived from





(*Left*) *Hemophilus influenzae.* Smear from culture of organism from meningitis; growth on chocolate agar, 24 hours. (Courtesy, Zinsser and Bayne-Jones: "Textbook of Bacteriology," 8th ed., New York, D. Appleton-Century Co., Inc.)

(*Right*) *Hemophilus influenzae.* Forms from R type colony. (Courtesy, Zinsser and Bayne-Jones: "Textbook of Bacteriology," 8th ed., New York, D. Appleton-Century Co., Inc.)

hematin. This is thermostable and acts like a peroxidase. The other factor, V, is a codehydrogenase, which is present in blood and also in certain vegetables, especially potato, and yeast. It is thermolabile. It is produced by certain bacteria, and it is for this reason that the influenza bacillus grows more luxuriantly in the neighborhood of colonies of other organisms (especially the staphylococcus, *G. tetragena,* etc.)—the so-called "satellite phenomenon."

Many strains produce indol, and all strains reduce nitrates to nitrites. Smooth (S) and rough (R) forms occur, and the transformation from S to R can be effected by cultivation. The R forms are less virulent and show more pleomorphism.

Serologic Classification. Six types (*a* to *f*) of *H. influenzae* have been described (Pittman, 1933, 1937) on the basis of precipitation of immune serum by capsular substance. At least 90 per cent of the strains found in the spinal fluid of patients with meningitis due to *H. influenzae* have been of type *b*. Types *a* and *f*, as well as non-type-specific strains, and *H. parainfluenzae* have been found in spinal fluid also. The non-type-specific strains and the unencapsulated (R) variants of the type-specific encapsulated strains are antigenically heterogeneous.



Colonies of *Hemophilus influenzae,* S and R types, after Pittman.

Typing of *H. influenzae* may be done by precipitation or by capsule swelling (Quellung reaction). Such tests may be done directly on spinal fluids or from cultures after growth is obtained. For precipitation the spinal fluid or fluid culture is centrifuged and the supernatant fluid is layered over the serum in a small tube. A "ring" of precipitate indicates a positive reaction. The Quellung test is performed in the same manner as for the pneumococcus or meningococcus.

Virulence. Influenza bacilli have, with some exceptions, very little virulence for animals, but when suspended in a solution of mucin and inoculated intraperitoneally into mice, type-specific strains, and also non-type-specific strains freshly isolated from pathologic sources, cause progressive fatal infections with bacteremia.

Occurrence. All types of influenza bacilli may be found in the healthy throat and nose. They may cause sinusitis, otitis media, angina, bronchitis, and bronchopneumonia, either alone or associated with some other organism. Non-type-specific strains have been found in tuberculous lesions of the lungs and in bronchiectatic cavities. Bacteria of this genus may cause a subacute bacterial endocarditis indistinguishable from that due to the *S. viridans.* A highly fatal type of meningitis in children is caused by influenza bacilli, usually of type *b*.

Relationship to Influenza. *H. influenzae* was originally isolated by Pfeiffer from the sputum and nasal passages of patients having influenza, and was believed

by him to be the etiologic agent of the disease. Recent work, however, has established the fact that a filtrable virus is the primary cause of the disease. Shope has shown that typical swine influenza can be produced in swine only when the influenza bacillus (*H. suis*) is inoculated together with the virus of influenza, and it is possible that a similar relationship may exist in man. On the other hand, the disease can be transmitted to ferrets by inoculation with the swine or human virus alone, and is not modified by the addition of either the human or porcine influenza bacillus.

Treatment of Infections Due to H. Influenzae. A specific antiserum for type *b*, prepared in rabbits and concentrated and refined, is available. The use of this serum, usually in conjunction with sulfonamides (i.e., sulfadiazine) has reduced the mortality in meningitis due to *H. influenzae*, type *b*, from over 90 per cent to 25 to 50 per cent. The value of antibiotics in the treatment of hemophilus infections has not yet been thoroughly established, but reports of the use of streptomycin are promising.

Hemophilus Parainfluenzae. *H. parainfluenzae* closely resembles *H. influenzae*. It is not type-specific and is less exacting in its growth requirements, as it requires only the V factor, and not the X. It is found in the healthy nose and throat as well as in infectious processes.

Hemophilus Hemolyticus. Certain strains of this organism require both X and V factors for growth whereas others require only V. Some of these tend to be very pleomorphic and often show long filaments or threads. Like *H. parainfluenzae*, this organism is found in the normal nose and throat and also in infectious processes.

Hemophilus Conjunctivitidis (*Koch-Weeks Bacillus*) (*Koch, 1883*). This organism produces a severe acute conjunctivitis which is highly contagious. It is particularly common in Egypt and the Philippines. The conjunctivae of animals, other than man, resist infection with this bacillus. Flies are an important factor in its transmission in Egypt. The incubation period of the disease is short, 12 to 36 hours.

Smears from the conjunctival secretion show large numbers of small Gram-negative bacilli, chiefly intracellular. They resemble *H. influenzae* morphologically and culturally, and are regarded by many as identical.

Moraxella Lacunatus (*Hemophilus lacunatus*) (*Diplobacillus of Morax*). This organism is no longer placed in the genus *Hemophilus*. It causes a mild chronic conjunctivitis, chiefly at the inner angle of the eye, and may produce a keratitis.

The bacilli are about 1 or 2μ $\times$ about 1μ in width, and tend to occur in pairs or short chains. They are nonmotile and Gram-negative. They grow only on media (preferably slightly alkaline) which are enriched with blood or serum. On Loeffler's medium they form, within 24 hours, little pits of liquefaction which tend to become confluent later. This is regarded as fairly characteristic. Neither the X nor V factor is necessary for growth.

Dialister Pneumosintes (*Bacterium pneumosintes*). During the pandemic of influenza in 1918 Olitsky and Gates obtained a minute, Gram-negative, anaerobic bacillus by inoculating rabbits intratracheally with material from the nose and throat of patients with early cases of influenza. Specific agglutinins of low titer were noted in the blood of the infected animals. On account of its small size, especially in young cultures, this organism may pass through the coarser bacterial filters. It can be cultivated on media enriched with fresh animal tissue and on blood agar plates under strictly anaerobic conditions.

This organism has also been isolated from the throats of healthy individuals (Branham, 1927) and there is no evidence of any etiologic relationship to influenza or other pathologic condition.

Chancroid

Hemophilus Ducreyi (*Bacillus of chancroid*) (*Ducrey, 1889*). This is often called the streptobacillus of Ducrey because it occurs in chains in cultures, although chain-formation is not commonly observed in smears made directly from lesions.

The organism is about 0.5 × 1.5μ, is Gram-negative, nonmotile, and forms no spores. It stains irregularly, often more intensely at the poles and often has the appearance of being constricted in the middle. It is found in the granulation tissue in the base of the ulcers and in the pus, often within leukocytes. Aspirating the gland juice with a hypodermic syringe from the buboes which are apt to follow chancroid offers the best means of obtaining pure cultures. This should be done before the bubo suppurates. Since Ducrey's bacillus is exceedingly delicate, the syringe should be warmed to body temperature and the transfer made to media at the same temperature and at once put in the incubator. Cultures kept at room temperature quickly die, but remain alive in the incubator for a week or more. Cultures and smears can also be made from the lesion, preferably before it ulcerates. Teague and Diebert got 140 positive cultures in 274 cases by the following method: Blood from a rabbit's heart was deposited in small tubes in 1 ml. quantities. After the blood had clotted, the tube was heated at 55° C. for five minutes to destroy natural bactericidal substances. The serum about the clot was inoculated with scrapings from the edge or floor of the ulcers or pus from a bubo and after 24-hour incubation smears were made and stained for small, Gram-negative bacilli in chains. Small, glistening, gray, pin-point colonies, which slide over the surface when touched with the platinum loop, may be grown by transfer to blood agar plates.

Intracutaneous diagnostic tests have been employed, using killed culture suspensions. Sanderson and Greenblatt (1937) report clinically correct results in 96 per cent of a series of about 75 cases and 100 negative controls. They introduced 1 ml. of sterile defibrinated human blood on to a tube of solidified beef infusion agar and inoculated this with 0.1 ml. of a growing culture. This was incubated 48 to 72 hours under reduced oxygen tension obtained by warming the tube to expel part of the air and sealing with paraffin. The blood was then removed and the sediment washed twice with sterile distilled water to remove hemoglobin. The sediment was suspended in salt solution, killed by heating 30 minutes at 60° C., and 1 : 10,000 merthiolate added. To make the test, 0.1 ml. was injected intracutaneously and the result observed after 48 hours. A positive reaction shows an indurated zone not less than 7 mm. in diameter, surrounded by an erythema of 14-mm. diameter. Bacillary suspensions of tested potency are now commercially available.

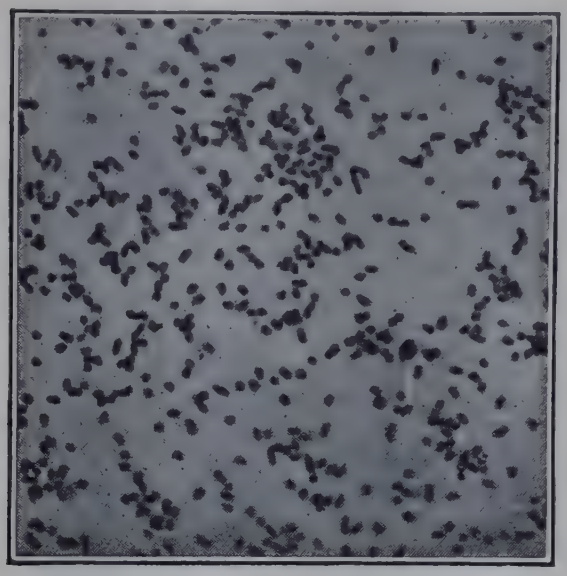
Whooping Cough

Hemophilus Pertussis (*Bacillus of Bordet-Gengou*). This bacillus was reported as the cause of whooping cough by Bordet and Gengou in 1906. It can be demonstrated by culture in the early stages of the disease from the secretions of the trachea, bronchi, and lungs.

Morphology. The bacillus is small (0.3 × 1.0μ) and ovoid, resembling the influenza bacillus. It is Gram-negative and nonmotile, occurring usually singly and in pairs, occasionally in short chains. Dissociated forms are more pleomorphic and may show filaments. Capsules can be demonstrated in freshly isolated strains.

Cultures. For primary cultures a special potato glycerin blood agar, a modification of

the original Bordet-Gengou medium, is used. Cultures can be made from the sputum in the ordinary way, or by holding a plate of the medium in front of the mouth of the patient during a paroxysm of coughing. Bradford (1940) recommends a nasopharyngeal swab, especially for young infants. The colonies develop rather slowly, at first resembling those of influenza bacilli, after 48 hours becoming slightly larger and more opaque. There is a narrow zone of hemolysis around the colonies. The bacillus does not ferment any of the carbohydrates. It remains viable, and grows at lower temperatures than the influenza bacillus. After cultivation on artificial media for a time, it can be made to grow on plain media, and, unlike the influenza bacillus, it does not require the V and X factors for growth.



Hemophilus pertussis. Organisms from 48-hour culture on Bordet-Gengou medium. (Courtesy, Zinsser and Bayne-Jones: "Textbook of Bacteriology," 8th ed., New York, D. Appleton-Century Co., Inc.)

Variation. Leslie and Gardner (1931) studied the antigenic properties of 32 strains, and divided them into four groups which they designated as phases 1, 2, 3, and 4. The freshly isolated (smooth) strains were of phase 1. Vaccines made from the S forms (phase 1) are considered more effective in immunizing. All recently isolated (smooth) strains appear to be identical in antigenic structure.

Vaccines. Vaccines made from the bacillus have been used extensively in prophylaxis and treatment. Their prophylactic value seems well established.

Laboratory Diagnosis. Laboratory diagnosis is of special importance in the early stages of infection before paroxysms have developed, or in atypical cases. Cultures are of greatest value. The high lymphocyte count associated with well-developed whooping cough may give valuable help in differentiating an atypical attack of pertussis from a persistent cough due to other causes. Direct microscopic examination of sputum is practically without value. Serologic tests—agglutination, complement-fixation, and opsonic reaction—on the patient's serum have diagnostic value after the disease is well established and in studying immunity resulting from natural infection or from vaccination. Intracutaneous tests for diagnosis have been used to some extent, but their value is not yet well established.

Both *H. parapertussis* (Eldering and Kendrick, 1937) and *Brucella bronchiseptica* (Brown, 1926) have occasionally been found responsible for cases clinically typical of whooping cough.

Brucellosis

Brucella Melitensis (*caprine type*) (*Bruce, 1887*); **Brucella Abortus** (*bovine type*) (*Bang, 1897*); **Brucella Suis** (*porcine type*) (*Traum, 1914*). Brucellosis primarily affects goats, cows, and hogs, causing abortion in cows; secondarily, these infections gain entrance to man, in whom the symptoms may be similar whether the infection is of goat, cow, or hog origin. The acute disease in man has been called undulant fever on account of the successive waves of pyrexia which may extend over several months.

Alice C. Evans, in 1918, discovered the close serologic relationship of the organisms of Bruce and Bang, thus laying the foundation for world recognition of the relationship of the three infections. Meyer and Shaw proposed the generic name Brucella in 1920.

Morphology. The three organisms cannot be differentiated morphologically, although freshly isolated *melitensis* cultures seem most like coccoid bacilli, averaging 0.3 to 0.5μ, while *suis* is the longest, and *abortus* is of intermediate length. All are Gram-negative, nonmotile, and non-spore-forming.

Cultural Characteristics. The organisms grow on ordinary laboratory media, especially well on glucose infusion agar, liver agar, or serum tryptose agar adjusted to a pH of 6.8 to 7.4 and incubated at 37° C. In primary cultures the colonies become visible in three to six days and are small, dewdrop-like, later becoming opaque and raised. In broth there is a diffuse turbidity. None of the carbohydrates is fermented. The organisms are killed by heating to 60° C.

Differential Characters. Likeness of the three species has stimulated intensive efforts to discover differences which would serve to identify a species irrespective of the host from which it was isolated. Differences have been sought in carbon dioxide requirements, serologic relationships, bacteriostatic action of dyes, hydrogen sulfide production, and glucose utilization.

increased carbon dioxide tension. *Br. abortus* was originally isolated by Bang who inoculated material from the uterus of an aborting cow into deep tubes of serum agar in which colonies developed only in a zone of partial oxygen tension 1 cm. below the surface. Huddleson found that atmospheric air containing approximately 10 per cent carbon dioxide was suitable for original isolation of *Br. abortus*. After freshly isolated cultures of *Br. abortus* have been subcultured a few times, they grow freely thereafter in ordinary atmospheric air, and the carbon dioxide requirement cannot be restored to them by long residence in an artificially infected animal. Furthermore a recently isolated *Br. abortus* culture cannot be made to lose its carbon dioxide requirement for isolation by long residence in a goat, which is the normal host of the air-growing *Br. melitensis*. Since normal strains of *Br. abortus* cannot be isolated originally in atmospheric air but only under increased carbon dioxide tension, the important differential character is established that a brucella organism which requires carbon dioxide for original isolation is *Br. abortus*. There are, however, strains of *Br. abortus* in cattle and man in Rhodesia which grow freely in normal air from the start, but the question arises as to whether they may not be vaccinal strains derived from living vaccine used for protecting cattle against contagious abortion. *Br. melitensis* and *Br. suis* do not require carbon dioxide for original isolation or for subsequent growth, although both species are susceptible of isolation in 10 per cent carbon dioxide and of subcultivation under increased carbon dioxide tension.

serologic relationships. The three species are agglutinated by an antiserum prepared from any one of the three; therefore the species cannot be differentiated by simple agglutination tests. *Br. abortus* cannot be differentiated from *Br. suis* by agglutinin absorption tests, but *Br. abortus* or *Br. suis* on the other hand can, in most instances, be differentiated from *Br. melitensis* by agglutinin absorption. However, there still remain a few *Br. abortus* cultures which cannot be distinguished by agglutinin absorption from *Br. melitensis*. Tularemia sera agglutinate the brucella oftener than brucellosis sera agglutinate *P. tularensis*, but in any case a correct differentiation between genera can be made by agglutinin absorption.

bacteriostatic action of dyes. Huddleson and Abell have been able to separate the three types by the inhibitory effect on growth which certain dyes exercise when incorporated into a culture medium of beef liver agar, as shown by the following table:

Table 10

BACTERIOSTATIC ACTION OF DYES ON THE BRUCELLAS

	Methyl Violet 1 : 100,000	*Basic Fuchsin 1 : 25,000*	*Pyronin 1 : 200,000*	*Thionin 1 : 50,000*
Br. suis	No growth	No growth	No growth	Growth
Br. abortus	Growth	Growth	Growth	No growth
Br. melitensis	Growth	Growth	Growth	Growth

HYDROGEN SULFIDE PRODUCTION. Huddleson, Hasley, and Torrey have shown that, depending upon the amounts of available sulfur in the culture medium, *Br. suis* produces hydrogen sulfide for the longest time, *Br. abortus* less, and *Br. melitensis* not at all. The test is made by suspending a strip of dry lead acetate paper in the culture tube above the liver infusion agar at the time of inoculation and observing the degree of darkening during four days.

GLUCOSE UTILIZATION. McAlpine and Slanetz have shown by chemical analysis that *Br. melitensis* and *Br. suis* utilize the glucose in the culture medium whereas *Br. abortus* does not.

CULTURES ISOLATED FROM MAN IN THE UNITED STATES. Human cases of brucellosis have been reported from all of the states, about 4500 being reported annually in recent years by state departments of health, principally on the basis of positive reactions to agglutination tests.

For a time *Br. melitensis* was isolated almost exclusively from residents of Texas, New Mexico, Arizona, or California, where goat-raising is a prominent industry. Infection from this organism has now spread until it is found in many states.

Br. abortus, because of its carbon dioxide requirement for isolation, has doubtless been missed in those laboratories in which carbon dioxide apparatus is not a part of the routine equipment used for blood cultures. Gilbert and Coleman working in the New York State Laboratory incubated duplicate samples of patient's blood and cow's milk in normal air and in an atmosphere containing 5 to 10 per cent of carbon dioxide. Of 88 cultures isolated (44 from human blood and 44 from cow's milk) all showed primary growth in the carbon dioxide environment, while practically none gave primary growth in normal air, thus indicating that only the bovine (abortus) type is prevalent in that state, where few hogs are raised. Huddleson isolated 57 cultures from man in Michigan, only two of which were *Br. suis.* The small number of these corresponds to the small extent of the hog-raising industry in Michigan.

Br. suis was the type in 69 of 104 cultures isolated from man in Iowa, 35 being *Br. abortus.* All were isolated by Hardy, Borts, and Jordan in the state laboratory where duplicate samples were incubated in air and carbon dioxide. The high percentage of *Br. suis* cultures corresponds to the prominence of the hog-raising industry in Iowa. Beattie and Rice reported a milk-borne epidemic of 30 cases in Council Bluffs, Iowa, confined to city users of raw milk supplied by one dairyman. *Br. suis* was cultured directly in normal air from the blood of 6 of the 30 patients and obtained from cream of the milk of one cow in the dairy by guinea-pig inoculation. This cow before purchase had associated with aborting hogs on another farm. *Br. suis* was the classification of 32 of 33 cultures isolated by Sellers of the Georgia State Laboratory from human blood, which were all incubated in normal air, none in carbon dioxide because of the lack of suitable apparatus. Whether the failure to isolate *Br. abortus* was due to the failure to use carbon dioxide apparatus remains a question. The epidemiologic evidence, however, points to drinking raw cow's

milk containing *Br. suis* as the cause. Evidence of the spread of *Br. abortus* from cows to hogs by contact is lacking, but the spread of *Br. suis* from hogs to cows by contact is amply demonstrated.

Pathogenicity for Laboratory Animals. In monkeys a disease resembling human brucellosis can be produced with caprine and porcine strains but less regularly with the bovine type. Ordinary laboratory animals are also less susceptible to bovine strains. Inoculation into guinea pigs (preferably males) may produce a low-grade infection of low mortality. After two or three months there is enlargement of the spleen, which is filled with small grayish, tubercle-like nodules. The liver and lymph nodes may also be affected, and there is often a caseous epididymitis.

Brucellosis in Man. The probability of the infection of man with *Brucella abortus* was suggested by Evans (1918) when she demonstrated its serologic relationship to *Br. melitensis*. The first human case was described by Keefer (1924), and a description by Carpenter (1927) of 10 cases established its clinical significance beyond a doubt. The disease is contracted by drinking raw milk from diseased cows, by contact with infected animals, or by handling infected meat. It may be spread by cheese and butter, since the souring of milk does not destroy the organism. Many laboratory infections have been recorded in laboratories in the United States and other countries. Infection may occur through the skin as well as through the alimentary tract. The spread of the disease through carriers has not been demonstrated. The results of agglutination tests on individuals with no history of the disease, especially veterinarians and butchers, suggest that an immunity may be acquired from subclinical or unrecognized infections. The incidence of *Br. abortus* infections is low in proportion to its frequency in cattle, and many of the cases are mild. The caprine and porcine types are more pathogenic for man.

The mortality from the disease is low, 2 to 3 per cent, but the sequelae may last for weeks or months or years. In many cases of melitensis infection there are regularly recurring periods of fever lasting from one to three weeks, but often quite uniform in the same individual. Each cycle is characterized by a preliminary period of daily steplike rise in temperature and sharp morning remissions; a period of high continued fever; and a period of steplike descent. These may follow each other in close succession for months, or they may be separated by varying periods of normal temperature. It is this cycle in some acute infections that is responsible for the term "undulant fever." A similar course is seen in some cases of abortus infection, but more often the temperature is irregularly elevated. Relapses are less frequent than in the melitensis cases. The pulse is relatively slow. The blood shows a leukopenia with a relative lymphocytosis. The spleen and sometimes the lymph nodes are enlarged. Muscle and joint pains are common. In long-continued cases there is a tendency to localization of the infection in some particular area. The spleen, lymph nodes, or intestinal tract may harbor the organisms after the general infection subsides. One or more joints may be involved, and the organisms have been cultivated from the synovial fluid. Suppurative lesions of the testicle, epididymis, ovary, gall-bladder, and appendix sometimes occur. Cases of meningitis have been reported in which the organism was found in the cerebrospinal

fluid. Neuritis, especially sciatica, is often noted. The early-morning sweats may suggest phthisis. These clinical features are not very distinctive, and the diagnosis depends upon laboratory investigations. Although positive reactions to laboratory tests are evidence of infection, negative reactions cannot be taken as proof of noninfection, especially in chronic cases. Obviously the diagnosis is frequently missed.

Laboratory Diagnosis. A positive *culture* is the one certain diagnostic finding. Very often cultures are not positive, especially in chronic cases. *Blood cultures* should be made at the onset of a febrile paroxysm. The organisms are present in the blood early in the disease in a large proportion of the melitensis cases, less often in the abortus cases. They may be present even after the fever has subsided. It is essential to incubate part of the culture in an atmosphere containing 10 per cent carbon dioxide if *Br. abortus* is suspected.

Methods for making these cultures are described in the chapter on blood cultures. Growth usually appears in from 5 to 10 days, but the cultures should not be considered negative for at least four weeks, since the primary growth of these organisms may be slow. Porcine and caprine strains are more easily isolated than bovine types and will grow both aerobically and under increased carbon dioxide tension. Hitchens has found that all gradations of oxygen tension may be obtained by adding 0.1 per cent agar to the fluid medium. Other means are used to secure the desired carbon dioxide tension.

The organisms can be obtained more regularly by *splenic puncture,* but the procedure is rarely justifiable.

Urine cultures are positive in some cases, especially when repeated examinations are made. These are usually made by spreading the sediment of the centrifuged urine sample over the surface of liver infusion agar plates containing 1 : 700,000 crystal violet.

The organisms have been cultivated from the *stools.* Amoss and Poston (1935) obtained cultures by adding an agglutinating anti-abortus serum to a suspension of the feces from which the gross particles had been removed. The agglutinated bacteria were then separated by a short centrifugation and plated out. The organisms have been grown also from local foci in the joints, gall-bladder, and meninges.

Guinea-pig inoculation is also used to obtain the organisms. The guinea pig is not, however, very susceptible to the bovine type. After about four weeks the pig is killed, and cultures are made from the spleen, blood, and lymph nodes. Agglutinins can be demonstrated in the serum. Special precautions should be taken by those handling the guinea pigs to avoid acquiring an infection.

Agglutination tests are relied upon for diagnosis later in the acute disease. After the fifth day agglutinins appear in the blood in a large majority of such cases. The test is performed with suspensions of caprine, bovine, and porcine strains as antigens. Serum dilutions are set up as usual in small tubes, using dilutions of 1:25 to 1:250 or higher. The antigen and serum dilutions are brought to room temperature before mixing, and must be incubated at least 42 hours before reading. Incubation is usually at 37° C., but temperatures up to 55° C. are acceptable. A titer of 1 : 40 is considered suggestive and 1 : 100 diagnostic, although it is often much higher. Proagglutinoid zones are common, and a number of dilutions should be set up. A rising titer has added significance. The agglutinins may persist for years after recovery. Many chronic cases never show agglutination. Negative reactions to agglutination tests do not mean that infection is not present.

The *opsonocytophagic test* (Huddleson, 1939) is of significance only in connection with other tests. A high index does not necessarily mean immunity, as a very high index has been found in fatal cases (Harris, 1943). It does indicate the degree of opsonic activity toward the brucellas.

Francis and Evans have pointed out that cross agglutinations occur with *P. tularensis,* and serum from suspected cases of brucellosis should be tested for agglutinins with this organism also. The titer of the serum will usually be higher for the homologous species, or, if both organisms are agglutinated to nearly the same extent, they can be identified by agglutinin absorption tests.

For the *intradermal test,* brucellergin (Huddleson, 1939) is the antigen of choice. This is an ether extract of the bacteria. A heat-killed *Brucella abortus* suspension may be used as antigen instead. Mixed strains may produce severe reactions. The test is done by injecting 0.1 ml. of brucellergin, or of the suspension containing 2000 million *abortus* organisms per ml., intradermally. The reactions are read at the end of two and four days. If negative, another reading should be made seven days later. A positive reaction indicates that the patient has at some time had a brucella infection. A diagnosis of brucellosis should not be based on this test alone. A negative reaction does not rule out brucellosis.

Treatment of Brucellosis. There is no specific or certain treatment for brucellosis. Vaccine therapy is advocated by some, but its value has not been definitely established. Drugs and antibiotics have not yet been proved of value. Prophylactic vaccination in man has not been practiced to an extent that permits conclusions to be drawn.

Tularemia

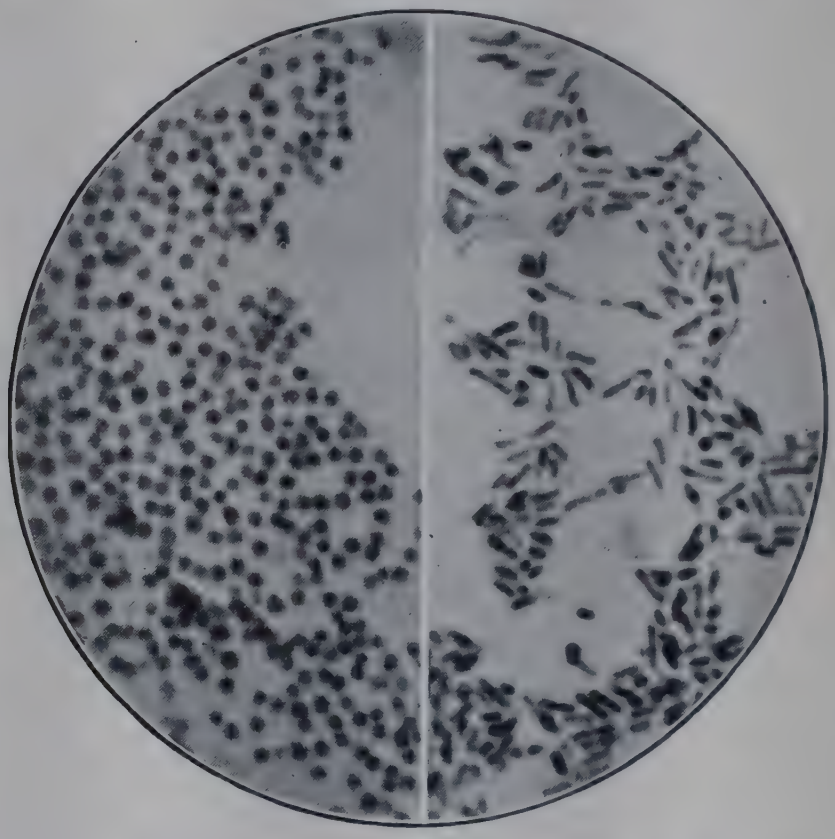
Pasteurella Tularensis (*Bacterium tularense*) (*McCoy and Chapin, 1911*). This organism has been found in more than 20 forms of wild life, especially rabbits and hares, and is transmitted to man by contact with the tissues of these animals or by the bites of various infected arthropods. Cases of tularemia have been reported from all parts of the U. S. except Vermont, and from Japan, Canada, Mexico, Russia, Norway, Sweden, Turkey, and Central Europe.

Morphology and Cultural Characteristics. *P. tularensis* is a very small, nonmotile, Gram-negative bacillus, from 0.3μ to 0.7μ in length. In fresh cultures, short bacillary forms occur; later coccoid forms predominate. Occasionally polar granules may be seen. In tissue smears the organisms appear to be surrounded by capsular material. They stain readily with ammonium oxalate crystal violet in smears from cultures and tissues of some animals, but Giemsa's stain is preferable for tissue sections. They may only rarely be demonstrated in tissues of man or guinea pigs.

They do not grow on plain media. They were cultivated first by McCoy and Chapin on a medium of the coagulated yolk of hen's egg. Francis found that the presence of cystine in the medium is necessary for their growth and has devised a glucose-cystine-blood-agar which is the most satisfactory medium for isolation and culture. Recently it has been shown that liquid media are capable of supporting growth of *P. tularensis.* The organisms may also be propagated on the chick embryo. (See section on media.) Francis has found that cultures kept for 12 years are still as exacting in their cystine requirements as when first isolated. On glucose-cystine-blood-agar colonies appear about the third day and are smooth, rounded, and opaque. The organisms are strictly aerobic and grow best at 37° C. The optimum reaction of the medium is pH 6.8 to 7.3. On account of their small size they will pass through some of the coarser bacterial filters. The organism causes fermentation with the production of acid but no gas in dextrose, glycerin, mannose, maltose, dextrin, and levulose.

These organisms are readily killed by heat (10 minutes at 56° C.); hence thoroughly cooked meat is not infectious. Francis has kept them alive and virulent in glycerinated guinea-pig spleen tissue at —14° C. for 10 years. No toxins have been demonstrated.

Infection in Animals. Wild rabbits, ground squirrels, wild rats and mice, opossums, grouse, and other animals have been found naturally infected. Tularemia is presumably transmitted from one animal to another by the bites of various arthropods and probably also by direct contact. The disease takes the form of a rapidly fatal septicemia with glandular enlargement and focal necroses in the spleen, liver, lymph nodes, bone marrow, and lungs.



Pasteurella tularensis. Note change from coccus-form to rod-form in a single transfer on culture medium. (Photomicrograph, Major G. R. Callender, M.C., U.S.A., by courtesy of Surgeon E. Francis, U.S.P.H.S.)

Transmission. In man, tularemia is acquired most commonly by handling the tissues of infected wild rabbits or other animals. The bacteria gain entrance through an abrasion in the skin or through the conjunctiva. It is believed that they may also penetrate unbroken skin. Other cases are acquired by the bite of the horsefly (*Chrysops discalis*) or of the wood ticks (*Dermacentor andersoni* and *Dermacentor variabilis*) which have become infective by feeding upon diseased rodents. Francis has found that in ticks the organisms may be harbored for long periods within the body cells and celomic fluid as well as in the lumen of the gut. Hereditary transmission has been demonstrated from infected adult ticks to their eggs, larvae, and nymphs. Many accidental infections of man have occurred in laboratories among those who have studied the disease. Ingestion of insufficiently cooked wild-rabbit meat causes a severe type of tularemia.

Clinical Manifestations. Clinically, tularemia is characterized by an irregular fever lasting two or three weeks or longer, with (usually) an initial local ulceration or a con-

junctivitis, and with swelling and, occasionally, suppuration of neighboring lymph nodes. The mortality is not high—under 5 per cent in recognized cases—but the constitutional disturbances may be marked and prolonged. Some patients are ambulant throughout. Four general types of the disease have been described, although in the opinion of some workers the division of clinical tularemia into these types is not justified.

1. Ulceroglandular. In this type there is an ulcerating papular lesion at the site of inoculation with enlargement and sometimes suppuration of the regional lymph nodes. This is the commonest form of the disease.

2. Oculoglandular. The initial infection is a conjunctivitis, and the neighboring nodes are enlarged.

3. Glandular. There is no primary lesion, but the group of nodes is affected which drains the site of infection.

4. Typhoid. Pyrexia occurs, but there is no local lesion or glandular enlargement. This type has been particularly common among laboratory workers.



Ulcer of finger 19 days after onset in a market man who dressed rabbits. (Brown and Hunter.)

PULMONARY MANIFESTATIONS. Bronchitis, pneumonia, a clinical picture of chronic tuberculosis, and pleural effusions have developed in a good many cases. Pulmonary or meningeal manifestations of tularemia may develop during the course of any of the above types, but are most frequently noted in the typhoidal cases. Pleural fluid from such cases agglutinates *P. tularensis* and by proper methods cultures of the organism may be isolated. In fatal cases the organism may be recovered from the consolidated lung at autopsy. In certain cases with pneumonic involvement the organism has been recovered from sputum during life, but this may also be accomplished with sputum from cases in which no signs of pulmonary involvement are manifest.

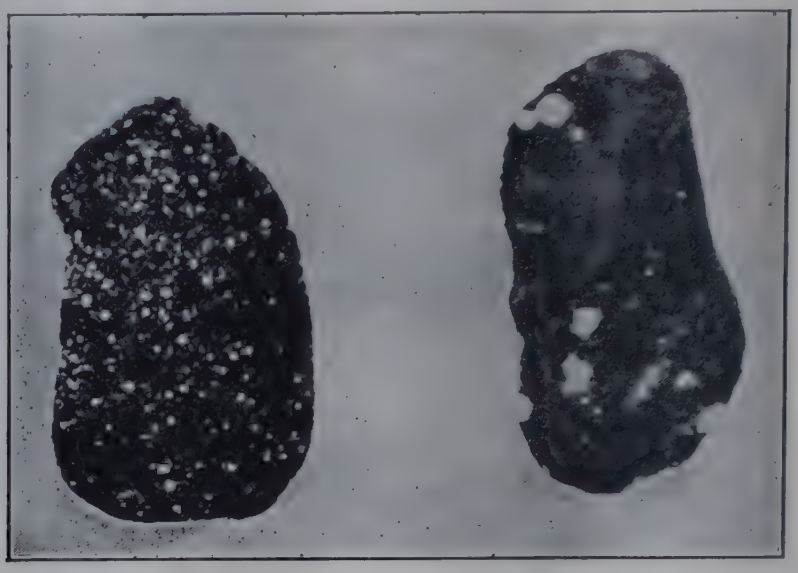
IMMUNITY. An attack of the disease confers a lasting immunity. However, one laboratory investigator who recovered from the disease 17 years ago has since had four transient local reinfections on a finger at different times without systemic disturbance.*

LABORATORY DIAGNOSIS. *P. tularensis* has been isolated from the blood early in the disease in a few instances, and from the local lesions and lymph nodes, but direct cultivation is difficult and impracticable for diagnosis. The most satisfactory method is animal inoculation. Material from the primary lesion, regional lymph nodes, sputum, pleural fluid, or blood is injected subcutaneously or intraperitoneally into guinea pigs, rabbits, or mice, which usually succumb within a week. At autopsy the animal shows hemorrhagic edema at the site of inoculation, caseation of the lymph nodes, and small necrotic foci in the spleen and liver. *P. tularensis* may be cultivated from the lesions and from the heart's blood. Glucose-cystine-blood-agar is inoculated with the blood or with small pieces of liver and spleen. Growth appears about the third day. Subcultures grow luxuriantly on this medium.

Infection can be produced in guinea pigs by cutaneous inoculation as in the case of the plague bacillus.

*Personal communication.

Agglutination tests are relied upon chiefly for diagnosis. Positive reactions appear during the second week of illness. A titer of 1 : 80 or over is considered diagnostic, particularly if the titer rises as the disease progresses. Titers as high as 1 : 82,000 have occurred, and a positive agglutination may persist for months or even years after recovery.



Guinea pig spleens, showing acute (*left*) and subacute (*right*) lesions of tularemia. (Francis.) (Army Medical Museum No. 40325.)

In the National Institute of Health the antigen is prepared by washing off a 48-hour growth on glucose-cystine-blood-agar with a small amount of saline containing 0.3 to 0.5 per cent formalin. The suspension is then thrown down in the centrifuge, and the bacterial sediment is taken up in saline containing 0.3 per cent formalin. This concentrated stock suspension is diluted at the time of use with saline. The agglutination is read after two hours incubation at 37° C., followed by overnight icebox storage. Nonvirulent cultures are suitable for preparing antigen. Such a culture may be obtained from the National Institute of Health, Washington, D.C.

Francis and Evans have pointed out that there is an antigenic relationship between *P. tularensis,* and *Brucella abortus* and *melitensis,* and that some tularemia sera contain group agglutinins for the latter organisms. Such tularemia sera agglutinate *P. tularensis* more quickly and to a higher titer than they agglutinate the brucella. If tularensis and brucella organisms are agglutinated to the same titer by a serum, the practical lesson is that a serum suspected of either tularemia or undulant fever should be tested against tularensis and against one of the brucella.

A skin test employing detoxified organisms may also be used in diagnosis. A uniformly positive reaction is obtained before the end of the first week of illness.

Treatment of Tularemia. Treatment has been largely symptomatic. Neither penicillin nor the sulfonamides appear to modify the course of the illness, but Foshay (1940) has developed a serum which appears to be beneficial, especially if administered early. Streptomycin is now used specifically for the treatment of tularemia (Hunt, 1947).

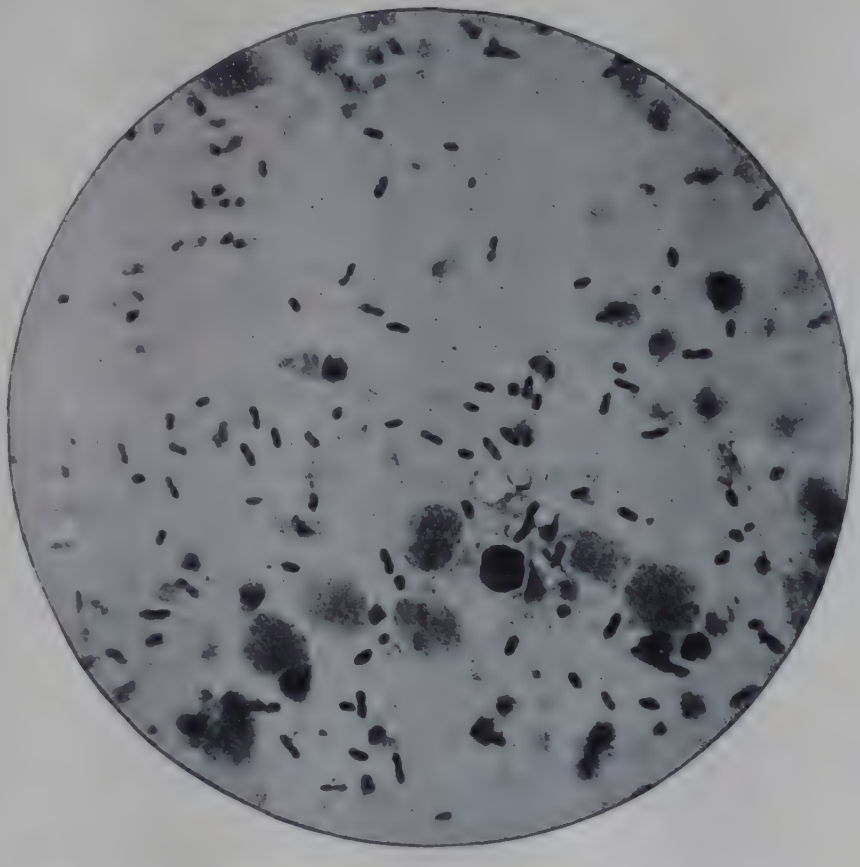
Gram-negative Bacilli Growing on Ordinary Media

Plague

Pasteurella Pestis (*B. pestis*) (*Kitasato, Yersin, 1894*). This organism is a member of the group of bacteria which cause the hemorrhagic septicemias (pas-

teurelloses) of various animals. Plague is primarily an epizootic disease of rats, but in some localities ground squirrels and other rodents have been shown to be the source of human infections. This was the terrifying "black death" of the fourteenth century which is believed to have killed 25 million people.

Morphology. In smears from the tissues, *P. pestis* is seen as a small coccobacillus ($0.5 \times 1.5\mu$) with rounded ends, occurring singly or in short chains of two or three. It is nonmotile and non-spore-bearing. It is Gram-negative and stains easily with any of the ordinary stains, and especially with Wayson's stain. When it is lightly stained, there is a characteristic bipolar staining which can be demonstrated better by fixing films with absolute alcohol than by heat. It is only in smears made directly from the tissues that this constant morphologic characteristic can be found. In old cultures, and particularly



Smear from a plague bubo, showing bipolar staining of *P. pestis.*
(From Lien-Teh, Chun, Pollitzer, and Wu: "Plague.")

in cultures on agar to which from 3 to 5 per cent of sodium chloride has been added, remarkable involution or degeneration forms which stain feebly are seen: swollen coccoid, root-shaped, or sausage-shaped forms, ranging from 3 to 12μ in length, and resembling molds or yeasts rather than bacteria. This peculiarity in morphology on salt agar is characteristic and of some value in identification. In tissue and in cultures grown at 37° C. a gelatinous capsule can be demonstrated. According to Schütze (1932) this envelope does not develop in cultures grown at 20° C., and only meagerly at intermediate temperatures.

Cultural Characteristics. *P. pestis* grows readily on the ordinary plain media. It is aerobic, and, unlike most pathogenic bacteria, grows best at a temperature of about 30° C. On agar plates the colonies are minute and transparent the first day, but by the end of the second day they become larger, opaque, grayish, and coherent. In older cultures there is so much difference in the size and appearance of the colonies that the culture appears to be contaminated. Marked acidity is produced in glucose but not in

lactose or saccharose broth. Ordinary broth cultures show a powdery or granular deposit at the bottom of the tube, and a hanging-drop preparation from such a culture may show chains of bacilli resembling streptococci. This deposit occurs most characteristically when the culture is incubated at room temperature in a medium without glucose. With glucose a spontaneous acid agglutination is apt to occur. If cultures are made in broth flasks containing drops of oil on the surface, a characteristic stalactite growth is often observed. Plague bacilli are readily killed by the ordinary disinfectants and by sunlight, but will withstand drying for at least a month. Francis has found cultures alive and virulent after seven years if suspended in glycerin and held at −15° C., and after nine years on agar if sealed and stored at 10° C. The virulence of these organisms varies under experimental conditions and in nature.

Plague in Animals. Plague is a natural disease of rats, and it has been found in ground squirrels and other rodents in this country. It has been suggested that the Siberian marmot or tarbagan (*Arctomys bobac*) may have been responsible for plague epidemics in the past. The disease is spread among rats by the bites of rat fleas from infected animals. A chronic form of rat plague has been described which may be a factor in perpetuating the disease.

Rats dead of the natural disease show characteristic gross pathologic lesions which, according to McCoy, are diagnostic. The most characteristic feature is the appearance of the lymph nodes. There is a marked hemorrhagic periglandular infiltration with caseation and often hemorrhage in the nodes. The subcutaneous tissues are congested and the pleural and peritoneal cavities filled with fluid. The liver is often sprinkled with small grayish-white granules. The spleen is swollen and congested, and often shows similar areas. This whole picture is not seen in all animals. In guinea pigs and rats the lungs often show lesions of various sizes each of which resembles a pearl set in coral. The appearance of all lesions usually depends a great deal on the length of survival of the animal after infection. If death occurs within two or three days there may be no discrete lesions, but only congestion and effusion. The bacilli are present in large numbers in the blood, spleen, lymph nodes, and other organs. If the carcass is decomposed, they may be isolated by inoculating a rat or guinea pig subcutaneously, or by rubbing the material vigorously on the freshly shaven skin.

Plague in Man. There are two chief types of plague in man: bubonic and pneumonic. In *bubonic plague* there may be a local lesion at the site of the bite, or this may be negligible. The regional lymph nodes become enlarged and exquisitely tender. In most of the cases the inguinal nodes are involved primarily, although in those which McCoy traced to ground squirrels the axillary nodes were most often affected. Later, secondary buboes develop, and suppuration may occur. There is usually a transient bacteremia during the first three days of the disease. In the later stages there is often a true septicemia. In *pneumonic plague* there is a fulminating and highly fatal bronchopneumonia or lobar pneumonia with great toxemia. The sputum is bloody and contains great numbers of the bacilli. The blood may contain them in such numbers (up to 500,000 per ml.) that they can be seen in smears (Strong and Teague). In *septicemic plague* there is a blood infection without localized lesions. A cellulocutaneous plague is also differentiated, in which the infection is located in the skin and subcutaneous tissues. The term *pestis minor* is sometimes applied to mild cases with glandular involvement but with only a slight constitutional reaction, in contrast to the typical disease, *pestis major*.

Transmission. Investigations in India determined definitely that bubonic plague is spread from rats and other rodents to man by the bite of the flea, *Xenopsylla cheopis*. In Europe and the United States, *Ceratophyllus fasciatus* is the common rat flea, and it as well as some other species of fleas may transmit the disease. Fleas suck blood from infected rats, and it has been shown that plague bacilli may develop in the proventriculus in such numbers as to cause obstruction. When the flea next bites there is a regurgitation, which carries the plague bacilli directly into the bite wound. The feces of the flea are

also infectious, and inoculation may occur through the skin by rubbing or scratching the bite. During epidemics the disease is spread from person to person by other fleas and by other biting insects. It has been shown experimentally that bedbugs may also transmit plague.

Pneumonic plague is spread by direct contact (droplet infection). Strong and Teague, studying the epidemic in Manchuria in 1910–1911, exposed plates in front of the mouths of these patients during a cough, and recovered plague bacilli in nearly 50 per cent of these cultures.

Laboratory Diagnosis. A guinea pig, mouse, or rat should be inoculated with the material. This may be emulsified and injected subcutaneously with a syringe, or a bit may be put in a pocket under the skin. Animals may also be infected by rubbing the material over the shaven skin. This latter method is especially applicable to contaminated material such as sputum. The ability to infect an animal through the unbroken skin has been regarded as a crucial test of the plague bacillus, but other organisms, notably *P. tularensis,* may be inoculated in this way. Death of plague-injected animals will usually occur within 10 days, and a typical pathologic picture will be found. A smear of the spleen stained with Wayson's stain shows characteristic bipolar organisms. Cultures from the spleen should yield a Gram-negative rod which produces acid, but no gas, from glucose, does not ferment lactose or sucrose, is methyl red-positive, indol-negative, Voges-Proskauer-negative, and reduces nitrates to nitrites. A number of sugars, other than those mentioned above, are reported to be fermented less constantly. The bipolar staining in tissues and the involution forms in old cultures and on salt agar are differentiating features of particular value.

The plague bacilli can be demonstrated in smears and cultures from the material aspirated from a bubo. In the later stages when softening begins they are less numerous or absent. In pneumonic plague the bacilli are found in the sputum. In septicemia cases and in very early cases (within three days of the onset) they may be grown in blood cultures. For precise identification of the organisms, agglutination tests with an immune serum should be carried out. Such agglutination tests are not always easy to perform as suspensions may either show a tendency to spontaneous agglutination or may be insensitive to agglutination. Adequate controls should be included. Several ways of preparing suspensions have been described by Wu et al. (1936).

Immunity. One attack of plague is believed to confer immunity. During the disease, agglutinins develop both in man and animals. Their demonstration is of some diagnostic value, although the titer is usually low. Agglutination in a dilution of a 1:5 or 1:10 is considered positive. Too much reliance should not be placed on agglutination unless positive and negative controls are included.

prophylactic vaccination. Prophylactic vaccination has been used extensively, especially by Haffkine in India, and both the incidence and the mortality of the disease have been greatly reduced. In the United States a somewhat less toxic vaccine is prepared by emulsifying the growth on agar slants and killing the organisms by formalin. Flu and others have used vaccines made from cultures dissolved with bacteriophage. Strong vaccinated a number of individuals with living

cultures which had been rendered avirulent. Otten and Girard and Robic have also used avirulent cultures in several million people with good results.

Schütze (1932) identified two antigenic constituents in plague bacilli: a somatic antigen and one occurring in the gelatinous capsule which is only present in cultures grown at 37° C. The former antigen is heat-stable and the latter heat-labile at 100° C. Schütze found that vaccines containing both antigens were much more effective in immunizing animals than those having only the somatic antigen.

Treatment of Plague. Therapeutic sera prepared by the injection of horses or cattle with living organisms have been used by Yersin and others. Variable success has been reported.

The sulfonamides have proved of definite value in the treatment of plague both experimentally and in the disease in man. Sulfadiazine has been found better than sulfathiazole. Penicillin has not been found effective in experimental infection, but streptomycin has given very promising results (Hornibrook, 1945; Wayson, 1946).

PASTEURELLOSES IN ANIMALS

Other *hemorrhagic septicemias* in various animals are due to organisms differing only slightly in cultural characteristics. They may be differentiated by their specific infectiousness and by serologic tests. Of these *Pasteurella pseudotuberculosis* (*Corynebacterium pseudotuberculosis,* Bergey) is closely related to *P. pestis* antigenically (the somatic antigen is common to both species, according to Schütze), but it has been shown by Arkwright to be motile under certain conditions. It is the cause of a fatal septicemia in guinea pigs, and occasionally causes confusion in animal experimentation. It is not pathogenic for white rats. *P. avicida* (*P. aviseptica*) of chicken cholera, *P. suilla* (*P. suiseptica*) of swine plague, and *P. cuniculicida* (*P. lepiseptica*) of rabbit septicemia and "snuffles" belong to this group. The name *P. multocida* is suggested to include the varieties *avicida, muricida, suilla,* and *cuniculicida. P. tularensis* produces somewhat similar lesions in guinea pigs, but rats are much less susceptible to this species than to the plague bacillus. They may be differentiated by serologic tests.

Glanders

Malleomyces Mallei (*Actinobacillus mallei, Bacillus mallei*) (*Glanders Bacillus*) (*Loeffler and Schütz, 1882*). This is the cause of a rather common disease of horses. When affecting the superficial lymph nodes it is termed "farcy"; when producing ulceration of the nasal mucous membrane it is called "glanders." The initial affection is usually followed by involvement of various organs, especially the lungs. Sometimes both lymph-node and mucous-membrane involvement occur in the same animal. Outbreaks have occurred in zoological parks among members of the cat family which were fed on horse meat. Cases have been reported in goats, sheep, and dogs, but cattle and swine are immune.

In man there are two types of glanders: chronic and acute. In the chronic form an abrasion becomes infected from contact with glanders material, and an intractable, foul, discharging ulceration results. Many nodules develop which form abscesses in the subcutaneous tissue, often ulcerating to the surface. There may be an involvement of the nasal mucosa as well as of the skin. This may persist for months with lymphatic involvement or may become acute. The acute form may also develop from the start, and the cases are usually regarded as pyemia. There is great

prostration with marked pains in the extremities. Pustular lesions, resembling those of smallpox, may be present. Acute glanders is almost invariably fatal.

Morphology. The bacillus is a narrow, sometimes slightly curved rod, varying greatly in size but averaging about $0.3 \times 3.0\mu$. It is nonmotile and Gram-negative. It often presents a beaded appearance, tends to stain irregularly, and may show clubbed ends. It stains readily, but is easily decolorized. In sections the bacilli are apt to be decolorized by the passage of the section through the alcohols.

Cultural Characteristics. The optimum temperature for growth is 37° C. and growth is slow. In primary cultures from pus or tissues the colonies may not appear for 48 hours. The growth is somewhat mucoid; later it becomes yellowish and more opaque. Glycerin agar and glycerinated potato are good media for isolation. Broth cultures show a slimy sediment and a surface pellicle. Milk is slowly acidified and coagulated. The characteristic growth is on potato. At first this is light brownish or yellowish, honey-like or mucilaginous. By the end of a week it has a cuprous oxide-like reddish tint with greenish borders, and the potato assumes a dirty brown color. Only *Pseudomonas aeruginosa* (*B. pyocyaneus*) and the cholera vibrio give a similar discoloration of potato, and these are easily differentiated.

Laboratory Diagnosis. Laboratory diagnosis is made by isolating and identifying the glanders bacillus culturally or by guinea-pig inoculation from the lesions. Smears for microscopic examination should be made of the pus from lesions, and stained with methylene blue. Blood cultures are usually negative. Cultures of glanders bacilli are among the most dangerous of all laboratory cultures, and should be handled with extreme care.

Animal Inoculation. If the material is injected intraperitoneally into a male guinea pig, marked swelling and often ulceration of the testicles occur within a few days (2 to 10) (the Straus reaction). Cultures should be made from the swollen testicle. The glanders bacillus can often be isolated from contaminated material in this way. Subcutaneous injection results in an involvement of the lymphatic system and the development of nodules—a picture suggestive of "farcy" in horses.

Strains of *M. mallei* from chronic cases are sometimes nonvirulent for guinea pigs, and diagnosis is dependent upon isolation in culture.

Mallein is a preparation analogous to tuberculin, and is used for the diagnosis of glanders in animals in a manner similar to that of tuberculin in the diagnosis of tuberculosis in man. It is rarely used in human cases. The reaction consists of a rise in temperature and local edema. Conjunctival instillation of mallein is also used.

Agglutination and complement-fixation tests are also employed for diagnosis.

Malleomyces Pseudomallei (*Bacterium whitmori*). This organism is the cause of a rare, glanders-like disease, described by Col. Whitmore of the British Army, found at autopsies of beggars in Rangoon. Stanton and Fletcher suggested the name "melioidosis," in order to describe its close relationship to glanders. *M. pseudomallei* closely resembles *M. mallei,* being a small Gram-negative bacillus about the same size and shape, and occurring in very large numbers in the acute lesions of the disease.

In culture it closely resembles the glanders organism, but it is motile, ferments some carbohydrates, and liquefies gelatin. It grows luxuriantly and forms a dense, wrinkled culture on glycerin agar. In guinea pigs the infection is more rapidly fatal than is glanders, producing in the male guinea pig an acute orchitis—the so-called Straus reaction. The organism is excreted in the urine and feces of infected laboratory animals. Several cases

of natural infection have been found in rats, and one case in a domestic cat has been observed by Stanton.

M. mallei and *M. pseudomallei* are closely related serologically.

Actinobacillus

For a time the glanders bacillus was classified as a member of this genus. Its few species are found in actinobacillosis of cattle, goats, and sheep. The most important species is *A. lignieresi,* a small Gram-negative, nonmotile rod which sometimes occurs in chains in liquid media. Its growth is favored by the presence of carbon dioxide, but it is not an anaerobe. It grows well on the usual enriched media. A characteristic granule formation occurs in cultures as well as in the thick pus of abscesses. The lesions in animals resemble those of actinomycosis to some extent, but are confined to the soft tissues, and do not affect the bony structures. The organism produces acid, but usually no gas, in dextrose and lactose. Milk is acidified but not coagulated. No indol is produced, and there is no nitrate reduction. The organism is chiefly of veterinary importance, although it has been reported in human infections.

Klebsiella

Klebsiella Pneumoniae (*B. pneumoniae, B. mucosus capsulatus*) (*Friedländer, 1882*). This organism is responsible for about 5 per cent of the cases of pneumonia, both lobar and lobular. Pneumonia due to this organism is severe, and often fatal. *K. pneumoniae* is also often associated with suppurative processes in the sinuses and middle ear. In children it may cause tonsillitis. It has been isolated from the blood, and from the spinal fluid in cases of meningitis. Recent reports indicate that treatment of these infections with streptomycin is often successful.

Morphology. The Friedländer bacillus is a short, thick rod, varying in size, but averaging $1.0 \times 2.5\mu$, nonmotile and Gram-negative. It has a large capsule which can be demonstrated in smears from pathologic material, such as sputum or animal exudates. The bacilli often occur in diplo-form, or in short chains or groups, surrounded by a continuous capsule.

Cultural Characteristics. The colonies on agar are large, semitransparent, whitish, and very viscid, later tending to become confluent. On potato the bacillus shows a thick, sticky growth containing gas bubbles. The distinctive cultural characteristic is the "nail" appearance in a gelatin stab. The growth at the surface is heaped up like a round-headed nail, the line of puncture resembling the shaft of the nail. The gelatin is not liquefied. Many of the carbohydrates are fermented with the production of acid and gas. No indol is formed. This latter characteristic helps to differentiate the organism from some of the colon bacilli with which it may be confused. If there is doubt, a mouse is inoculated intraperitoneally. Death from septicemia occurs within two days, often within a few hours, and the encapsulated bacilli can be found in the sticky peritoneal exudate, and in the blood and organs.

The Friedländer bacillus resembles closely *Aerobacter aerogenes.*

Serologic Types. Three distinct serologic types, A, B, C, have been differentiated by Julianelle among strains from various sources. He designated other apparently unrelated strains, not falling into these types, as group X. Most of the type A strains are of human origin; type B includes strains largely from lower animals. Type-specificity is dependent upon the carbohydrate haptene in the capsule. The carbohydrate of the Type B Friedländer bacillus resembles chemically and serologically that of the type 2 pneumococcus, and a Friedländer antiserum protects mice against this pneumococcus and vice versa. The specific soluble substance has been demonstrated in the urine of patients with a Friedländer pneumonia by precipitin tests with immune sera.

Julianelle dissociated smooth, encapsulated, virulent organisms into rough, nonencapsulated, nonvirulent forms. The latter contain only the group antigen in the cell body, and immune sera prepared with them will agglutinate all organisms of this species.

Enteric Group of Bacteria

This group includes a large number of morphologically similar, Gram-negative, non-spore-forming, usually nonproteolytic bacteria which are found in the intestinal tract of man and animals, some being normal saprophytes and others pathogenic. The term "enteric" can be misleading, since there are many other bacteria found in the intestinal tract that are otherwise designated. The enteric group includes the typhoid and dysentery bacilli, the salmonellas, and the coliform organisms. Since they are morphologically indistinguishable, great reliance is placed upon cultural and serologic means of identification.

Methods of isolation and study of the typhoid, dysentery, and salmonella organisms have much in common, and can be discussed in a general way for all of these bacteria before discussing each genus separately.

Materials for Examination. *Feces* are usually collected by means of rectal swabs. A cotton-tipped applicator may be used alone for infants; for adults it is best to slip the swab into a small rubber tube with an end cut on a bevel. This tube is lubricated and slipped into the rectum past the sphincters. If it is necessary to ship the material, a small amount of feces may be placed in a 1-ounce screw-cap glass bottle one-half full of 30 per cent glycerin in normal saline which has been buffered with disodium phosphate to the desired pH, usually about 7.5. Bromcresol purple may be added to indicate changes in pH.

Urine specimens are preferably collected with a catheter. If a voided sample is submitted, an equal volume of 30 per cent glycerin should be added. Urine examinations are made chiefly when typhoid fever is suspected.

Whole *blood* is taken from a vein into a sterile tube and allowed to clot. The clot is used for cultures, and the serum may be used for serologic tests.

Less often *duodenal contents* and *gall-bladders* removed surgically are examined for the typhoid bacillus. For shipping, the former should be placed in a small, tightly stoppered or sealed glass container, and gall-bladders should be placed in a container with 30 per cent buffered glycerin solution.

Occasionally samples of *food* from outbreaks of food poisoning are examined for salmonella organisms or staphylococci.

Sewage is also examined more frequently than formerly.

All of these materials are examined for the typhoid bacillus and for some of the salmonellas. Usually the dysentery bacilli are found only in feces, as they have less tendency to cause generalized infections.

Bacteriologic Examination. With some materials a preliminary incubation in a selective *"enrichment medium"* is indicated. Selenite-F medium (p. 353) is the commonest of these and tetrathionate solution (p. 352) is also good. Incubation in these for 12 to 24 hours increases the ease of isolation of the typhoid bacillus and of the salmonellas, but is less successful with the shigellas.

The choice of medium for "plating out" depends upon the material to be studied and upon the purpose to be accomplished. For typhoid, bismuth-sulfite agar (Wilson and Blair) (p. 355) is a highly selective medium which markedly inhibits most of the coliform bacteria. Undiluted feces, raw sewage, and rectal swabs can be streaked directly upon plates containing this agar. The typhoid colonies are flat and black, usually surrounded by a dark halo which gives a metallic cast to the medium in reflected light.

Many salmonellas grow well upon this medium, forming larger and more convex colonies than does the typhoid bacillus. However, salmonellas do not always do well upon this medium.

For salmonellas and the shigellas a bile salts citrate agar, commonly called the "SS" agar (Salmonella-Shigella) (p. 354) is good. Here, too, the selective nature of the medium permits direct inoculation with undiluted material. The rectal swabs usually taken for the shigellas can be "painted" all over the surface (Hardy, 1944) of the plate. Colonies of typhoid, shigella, and salmonella bacteria are translucent, colorless, or a delicate pink. Colonies of the coliform organisms are deep pink to red and are opaque.

The highly inhibitory properties of bismuth-sulfite and "SS" agar often make it desirable to use a second less selective plating medium such as MacConkey's bile salt agar (p. 354). On this medium the coliform organisms are brick red and the typhoid, shigellas, and salmonellas are colorless.

Sometimes it is desirable not to inhibit the coliform organisms. A medium that is differential without being inhibitory is the Endo agar used for many years (p. 353). The coliform bacteria show red colonies with a metallic sheen; non-lactose-fermenting colonies are colorless.

After 16 to 24 hours of incubation the plates are examined under a dissecting microscope or with a hand lens. Carefully chosen colonies are fished to slants of Russell's double-sugar or Kligler's iron agar (p. 356). Growth from the colonies is streaked on the slant and "stabbed" into the butt of the medium. After incubation the typhoid and shigella organisms will show an acid butt and unchanged slant; the salmonellas show both acid and gas in the butt; members of the coliform group show acid and gas throughout. The advantage in using Kligler's iron medium is that hydrogen sulfide production is indicated also by a blackening of the medium. In studying coliform or proteus strains, similar slants of "TSI" (triple-sugar-iron) containing saccharose also (p. 357) are useful.

Further identification may now be carried out by studying fermentation reactions in sugar broths and by testing for indol production (p. 352), acetyl-methyl carbinol production (Voges-Proskauer, p. 350), urea splitting (p. 351), gelatin liquefaction (p. 349), reaction in bromcresol-purple milk (p. 347), and by agglutination with appropriate sera. Not all of these tests will be indicated for all cultures studied. An excellent medium for studying fermentation is a meat extract broth with indicator, to which the desired carbohydrate is added aseptically in sterile aqueous solution after the medium has been sterilized (p. 342). A simple semisolid medium plus carbohydrate and indicator is preferred by some (Enlows, 1923) (p. 343).

The most important biochemic characteristics of the enteric organisms are summarized in a table on the inside of the back cover of this book.

The Typhoid Bacillus

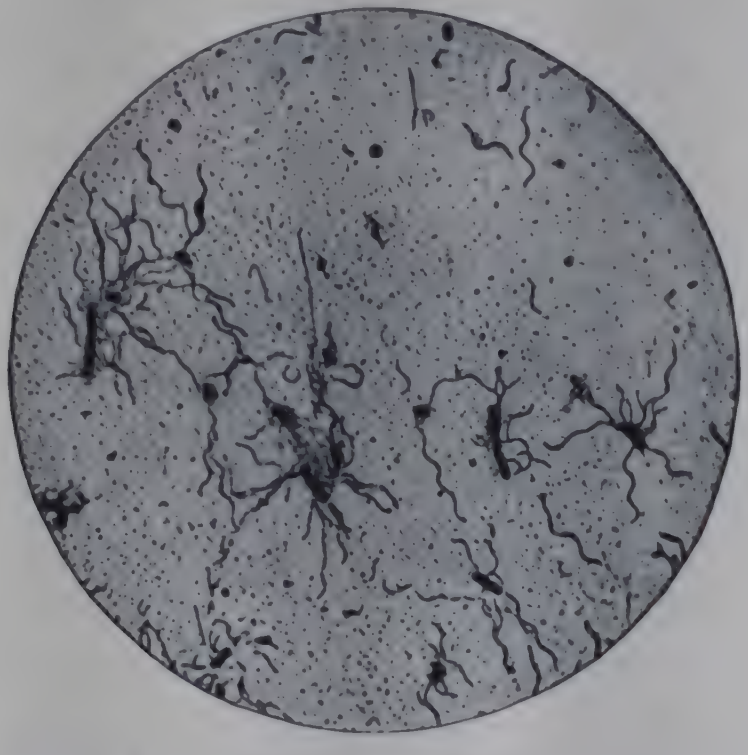
typhoid fever

Salmonella Typhi (*Eberthella typhosa, S. typhosa,* Bergey's Manual) (*Eberth, 1880; Gaffky, 1884*). This organism is the cause of typhoid fever.

Morphology and Cultural Characteristics. The typhoid bacillus is usually about $0.6 \times 3.0\mu$. It is more slender and more actively motile than members of the colon group. It grows readily on plain media, but its growth is more delicate and transparent than that of the colon bacillus. Its growth on potato is delicate, translucent, and glistening, and it does not discolor the potato as does *Escherichia.* Broth is diffusely clouded. Milk is at first slightly acidified, later becoming alkaline; it is not coagulated. The bacillus ferments dextrose, forming acid but not gas, and does not ferment lactose or saccharose. Acid is produced from a number of other sugars also. For its action on the more important of these carbohydrates the table on inside of the back cover of this book may be

consulted. It does not produce indol. Both Eberthella and Shigella strains produce a similar reaction on Russell's double-sugar agar (p. 356) (acid without gas in the butt). Important aids in differentiation are the motility of the typhoid bacillus, its fermentation of sorbitol, and its production of hydrogen sulfide. On Kligler's iron agar (p. 356) this last property is also shown.

Variation of the organisms of this group is of the greatest practical importance. Variation in the physical characteristics of the colonies (smooth and rough) occurs, and motile and nonmotile varieties of either S or R forms are encountered. Arkwright describes the following chief variants of the typhoid bacillus: (1) smooth, motile; (2) smooth, nonmotile; (3) rough, motile; and (4) rough, nonmotile. These types differ antigenically.



Bacillus of typhoid fever, stained by Loeffler's method to show flagella. (× 1000.) (Williams.)

There are three different types of antigen of practical importance in the typhoid bacillus.

1. The flagellar or H antigen derived from the flagella, which produces the large-flake agglutination with its antiserum. This antigen is heat-labile at 80° to 100° C., and is destroyed by 50 per cent alcohol. An alcoholized or heated suspension of the organisms, therefore, contains only the following O antigen. The H antigen and antibody have little relation to protection.

2. The somatic or O antigen, contained in the body of the organism, which produces a small-flake or granular agglutination. This antigen is heat-stable, resists boiling, and is destroyed by formalin. A formolized suspension, therefore, contains only the H antigen.

3. The "Vi" antigen (Felix and Pitt, 1934) is found only in virulent strains. It is probably somatic, and it masks the O antigen so that the strains containing it are inagglutinable by O sera. This Vi factor, associated so closely with virulence,

is easily lost. This explains why so many freshly isolated strains of the typhoid bacillus have been found inagglutinable at first, but later were agglutinated well. The Vi antigen is present only in living, unheated cultures. It will withstand 56° C. for a short time, and is weakened by formalinization.

In the study of the antigenic relationships of this group of organisms it is necessary to investigate these antigens separately, in addition to making the ordinary agglutinin absorption tests, since some have a common somatic antigen with different flagellar antigens, and others a similar flagellar antigen, but different somatic antigen. For instance, the O antigen of the typhoid bacillus is similar to that of *S. enteritidis,* while their H antigens are quite different. Likewise the H antigen of *S. typhi* appears to be the same as the H antigen of the Stanley type of Salmonella in its specific phase. The problem is relatively simple in the case of the typhoid bacillus, since the H antigen is monophasic and specific—that is, not variable in its serologic reactions, as is that of some of the paratyphoid bacilli. The typhoid bacillus is placed in the genus *Salmonella* because of these antigenic relationships.

Pathogenicity. Typhoid fever cannot be produced in animals except perhaps in the chimpanzee. It is possible, however, by intravenous injection of the bacilli to produce in rabbits a local infection of the gall-bladder which may persist for weeks—a chronic carrier state. Large doses of living or dead bacilli may kill an animal through the action of the endotoxin. Mice can be infected by intraperitoneal injections of organisms suspended in mucin. (See Prophylactic Vaccination, p. 128.)

Infection. The organisms enter the body through the alimentary tract. During the period of incubation, which is usually 7 to 14 days, they multiply in the lymphoid tissue of the intestine, particularly in the Peyer's patches, and later invade the lymphatics. The adjacent lymph nodes, and in particular the spleen, become involved. After a time, which is approximately the period of incubation, the bacilli become so abundant that they are carried over into the general circulation, and a transient bacteremia results. When this happens bacteriolysis of the organisms occurs, with liberation of the endotoxins and the development of symptoms. This invasion of the blood stream occurs early in the disease in practically all cases. The bacilli usually reappear in the blood at the onset of a relapse.

As a result of the formation of antibodies the typhoid bacilli gradually disappear from the blood, and become localized in other areas. They are present in the gall-bladder, probably in all cases. They may produce no symptoms, or a cholecystitis may result. Bile is a favorable culture medium, and they may persist here for years. They have been cultivated from the center of gall-stones many years after the original attack of typhoid fever.

After the first week the bacilli increase in number in the stools, and are more easily found in cultures. This increase in number is due not to multiplication within the lumen of the intestine, but to discharge into the intestine of infected bile, and probably to ulceration of the Peyer's patches. Frequently they may persist for years in the stools, and the person becomes a chronic carrier.

During this period of the disease the bacilli are often (25 to 30 per cent) found in the urine. Transient periods of bacilluria are frequent. The bacilli may cause no lesions; or suppurative conditions in the kidney, pyelitis, or cystitis may follow. In either case the organisms may remain in the urine for years.

In patients with an associated bronchitis or bronchopneumonia the bacilli may be found in the sputum.

Localized lesions elsewhere due to the typhoid bacillus alone or in combination with some other organism may occur late in the disease—periostitis, osteomyelitis, or deep abscesses. The bacilli have been found in the cerebrospinal fluid in typhoid patients with meningitis. They have been demonstrated in the lymph spaces of the rose spots.

After the first or second week demonstrable antibodies develop in the blood—bacteriolysins, opsonins, precipitins, and agglutinins. The demonstration of agglutinins in the blood is the most important diagnostic procedure after the bacteremia has subsided. Agglutinins may not appear until convalescence, however, and fail to develop in about 5 per cent of the cases.

Carriers. Although most typhoid fever patients become bacteria-free after about three months, a certain number (2 to 5 per cent) develop into chronic carriers and may remain so for many years. Most of these carriers harbor the organisms in the gall-bladder, some in the intestinal tract only, and some in the urinary passages. The bacilli have been found in the feces of contacts who did not contract the disease. Dissemination of the bacilli by these carriers is the great factor in perpetuating the disease. Large epidemics have been caused by pollution of the water supply. Contamination of food, especially milk, by carriers or by flies has resulted in outbreaks. The chlorination of water supplies and better methods of sewage disposal have greatly lessened the incidence of typhoid. The detection of carriers, especially among individuals who handle food, is of great importance. The treatment of carriers seems to be of little use. In cases in which the gall-bladder is shown to be the source of the infection, cholecystectomy offers the best chance of relief from the carrier state.

Laboratory Diagnosis: blood cultures. During the first week of the disease typhoid bacilli can be isolated from the blood in about 90 per cent of the cases. Positive cultures are obtained in from 70 to 60 per cent in the second and third weeks, and after that time the percentage falls further. For methods of culturing the blood the chapter on blood cultures should be consulted. The organism should be identified culturally, and by agglutination with appropriate antisera. For study of fresh virulent strains a serum prepared with a pure Vi strain should be included. It is desirable to absorb such a serum with a strain of *S. typhi* known to possess little or no Vi antigen. An antiserum containing the usual H and O agglutinins should also be used and it is well to remember that the somatic O antigen of *S. typhi* is apparently identical with the O of *S. enteritidis*.

stool cultures. Stool cultures are occasionally positive early in the disease, but after the first week the typhoid bacillus may be isolated in most of the cases. The material is streaked over bismuth sulfite or MacConkey's agar plates. When it is practicable to pass a duodenal tube, the typhoid bacillus can be isolated more readily by making cultures from the bile in the same way. Identification by serologic as well as cultural methods is necessary.

urine cultures. Cultures of urine are positive in about 25 per cent of the cases after the second week. The urine should be centrifuged, and the sediment cultured, or broth flasks heavily inoculated. Plates of special media should be used unless the urine is obtained with aseptic precautions.

No patient should be released from isolation until cultures from the urine and feces after catharsis, and preferably from the bile also, are negative. These methods are also used to detect carriers.

widal test. After the second or third week the Widal test is the chief diagnostic aid. This should be repeated at intervals in order to detect any increase in the titer of the agglutinins. The technic of the microscopic and macroscopic agglutination test, and methods of preparing the antigens are described in the chapter

on immunity and hypersensitiveness. Cultures of known agglutinability must be used. A formalin-treated suspension for demonstrating floccular H agglutinins, an alcohol-treated suspension for demonstrating granular O agglutinins, and a pure Vi suspension should be used. The living Vi suspension should be freshly made each time a test is performed. If only one antigen can be used the formalinized one is chosen. Since typhoid and the paratyphoid fevers may be indistinguishable clinically, it is necessary to test the agglutination of these paratyphoid strains also with the serum. Some group agglutination of these organisms is very common in the higher concentrations of the serum, but it is less marked than that with the typhoid bacillus, and is unusual in dilutions of over 1 : 40.

In individuals who have not received typhoid vaccine, agglutination with a 1 : 50 dilution of serum justifies a strong suspicion of typhoid fever, which is confirmed if the titer rises as the disease progresses. Agglutination in a dilution of 1 : 100 with O antigen or more with H antigen is regarded as significant. Carriers may show some slight agglutination, but there is no important change in the titer of the serum on successive examinations.

If, however, the individual has previously received typhoid vaccine, difficulties arise in the interpretation of a positive reaction to the agglutination test. Within a few days after vaccination the serum will usually show a high titer of specific agglutinins. This is followed by a fall in titer, at first rapid and later very gradual, so that over a short period the titer is practically unchanged. If such an individual develops typhoid fever the titer of the serum will increase as the disease progresses. This, however, may occur also in infections other than the typhoid fevers, as for instance in infections due to the pyogenic cocci or other bacteria—the so-called "anamnestic reaction." One can only conclude that the results of the Widal reaction in vaccinated individuals must be interpreted with the greatest caution, even when the titer is high and rises with the progress of the disease.

Prophylactic Vaccination. The value of prophylactic vaccination as originally introduced by Wright has been amply demonstrated.

For many years most of the typhoid vaccine used in this country and in England was made from a single culture, "Rawlings," isolated by Wright in 1900. Grinnell's investigations with mouse protection (1932) led to the conclusion that virulent smooth strains should be substituted for the Rawlings strain for the production of vaccine, and that the demonstration of its ability to produce H and O agglutinins is not an adequate measure of its immunizing power.

This question was investigated by Colonel Siler and his associates of the Army Medical Corps in an attempt to increase the protective properties of the typhoid vaccine used in the U. S. Army. Their strain No. 58 is now used in the preparation of the new vaccine. This is a smooth variant with high virulence and immunizing power, as tested by the production of active immunity in mice and protective power for mice of the serum of vaccinated individuals. The Army method of preparing and administering the vaccine is quite generally followed. The vaccine is prepared by growing the culture upon a solid medium for 18 hours, washing the growth off with normal saline, killing the organisms by heating at 56° C. for one hour, adjusting the density to 1000 million bacteria per ml., adding 0.5 per cent phenol as a preservative, and testing the final product for sterility. The immunizing potency of the vaccine may be tested by the injection of suitable mice which are subsequently given a "challenge dose" of virulent typhoid bacilli suspended in mucin (Griffitts, 1944). The usual method of administration is three injections a week apart of 0.5 ml., 1.0 ml., and 1.0 ml. For revaccination, one dose of either 0.1 ml. intracutaneously or 0.5 ml. subcutaneously seems adequate.

Much of the typhoid vaccine used is of the "triple" type, containing 250 million organisms each of "paratyphoid A" (*Salmonella paratyphi A*) and "paratyphoid B" (*S. paratyphi B*). A more severe reaction is often associated with the injection of this triple vaccine than with that made with typhoid alone.

The employment of agglutination titer as a measure of immunity is unsatisfactory. There is a marked difference in titer in different individuals after vaccination, and in a given individual, after reaching a peak in about 30 days, the agglutinin titer falls rapidly. Immunity may exist in the absence of agglutinins.

A mouse-protection test is a more satisfactory measure of immunity (Grinnell, 1932; Rake, 1935).

Neither vaccines nor therapeutic sera have proved to be of value in the treatment of typhoid fever, nor has the usefulness of antibiotics been established.

Salmonella Group

This is a complex group of organisms responsible for a variety of disorders in man and animals. In man some members cause a disease resembling typhoid fever (paratyphoid fever); others cause acute gastroenteritis, usually associated with the ingestion of infected foods; some members have a tendency to cause generalized infections which may subsequently localize as in a meningitis. A large number of species are found chiefly in animals. All must be regarded as at least potentially pathogenic for man.

These organisms are alike morphologically and culturally on ordinary media. The appearance of the colonies on selective and differential-plating media has been described (p. 124). The salmonellas may be differentiated roughly from the colon group by their inability to ferment lactose and saccharose, and from the typhoid and dysentery organisms by their ability to ferment dextrose and mannite with gas production. The individual types react alike on a Russell tube, producing acid and gas in the butt, and no change on the surface of the slant. As a rule a culture may be considered to fall into the salmonella group if it shows the following characteristics: ferments dextrose with both acid and gas, but not lactose or saccharose; produces a slight acidity in milk followed by a marked alkalinity in four to seven days; does not produce indol or liquefy gelatin, and does not produce acetyl-methyl carbinol. For other biochemic reactions the discussion of the individual members of the group and the table on the inside of the back cover may be consulted. The typhoid bacillus does not produce gas. Although it is now included in the genus *Salmonella,* it has been discussed separately.

Antigenic Structure and Variation. As with the typhoid bacillus the other salmonellas have different types of antigens. There are somatic O antigens which are heat-stable and formalin-sensitive. There are also flagellar H antigens, heat-labile and resistant to formalin. The Vi antigen has been found in a few types but is not a common component of the salmonellas. There may be several O antigens in each salmonella strain; also each of these O antigens may occur in some other salmonella strains.

This complex antigen situation is further complicated by the fact that some salmonellas are diphasic. The somatic O antigens remain the same, but the flagellar H antigens are different according to whether the culture is in phase 1 or

phase 2. In phase 1 the organisms are agglutinated only by a homologous serum; in phase 2 they are agglutinated by both homologous and heterologous sera. It is evident that much confusing cross-agglutination will occur with sera prepared with phase 2 cultures. Some salmonella types are monophasic. Usually both antigens are present in diphasic strains, but one may predominate.

For many years the classification of the salmonellas was in a very confused state. The work of Andrewes (1922, 1925) on rough (R) and smooth (S) variation, which occurs in the salmonellas, and that of Weil and Felix (1920) on H and O antigens formed the background for the method of antigenic analysis of the salmonellas as developed by Bruce White (1929) and Kauffman (1941). In this schema the O antigens are designated by Roman numerals; the H antigens in the specific phase 1 are assigned small letters, and those in the nonspecific phase 2 are given Arabic numbers. Thus, the complete antigenic formula for one of the strains of *S. paratyphi B* is I, IV, V, XII: b-1, 2; that for phase 1 is I, IV, V, XII: b; that for phase 2 is I, IV, V, XII: 1, 2. In some other strains certain O antigens may be lacking.

Salmonella strains have been placed in groups according to the dominant O antigen; these groups are designated by capital letters, but the letter does not appear in the formula. All members of a group have at least one O antigen in common.

In the serologic examination of salmonella cultures the O antigens are first identified, using O sera representing various heat-stable antigens of the subgroups. All major antigens in a strain must be determined in order to place it correctly. This may be done by means of 26 O sera and 39 H sera which have been carefully chosen and absorbed for that purpose. Actually, most of the strains commonly encountered can be differentiated by the use of relatively few of these antisera. Obviously it is impracticable for every diagnostic laboratory to prepare its own sera for this exacting work. Salmonella-typing centers have been established to carry out this identification and cultures are preferably sent to these places for study. The International Salmonella Center, of which Kauffman is director, is at the State Serum Institute in Copenhagen, Denmark. Type cultures and representative sera are sent from there to centers all over the world. The chief center in the United States is at the Agricultural Experiment Station, Lexington, Kentucky, under the direction of Edwards. Information concerning the location of other centers may be obtained from these stations.

More than three hundred types of salmonellas have been given formulas and names. Many are very closely related. New varieties and types are frequently found. To illustrate the complexity of structure of these microörganisms a few are listed in Table 11 (p. 132).

For the novice, biochemic reactions and the use of a few sera prepared from smooth cultures in a specific phase are very helpful in diagnosis. A few of the most important and representative species are discussed below.

These include the three most commonly found in paratyphoid fever and those long known to be associated with gastroenteritis, or "food poisoning."

PARATYPHOID BACILLI

Clinically, paratyphoid fever bears a close resemblance to typhoid fever. It may be caused by *S. paratyphi A*, *S. paratyphi B*, or *S. paratyphi C*.

Salmonella paratyphi A (paratyphoid A) (*S. paratyphi*, Bergey's Manual) is distinguished from other members of the salmonella group by its inability to ferment xylose, or to form hydrogen sulfide, or to produce a strongly alkaline reaction in milk. *S. paratyphi A* is monophasic (phase 1) and strains of this group A are serologically homogeneous.

Salmonella paratyphi B (paratyphoid B) (*S. schottmuelleri*, Bergey's Manual) produces acid and gas from xylose, produces hydrogen sulfide, and eventually renders milk strongly alkaline. Strains in this group B vary in their antigenic properties, and may be very difficult to classify. *S. paratyphi B* is readily differentiated from *S. paratyphi A* but has confusing cultural and serologic relationships with other salmonellas of group B which are associated with food poisoning.

S. paratyphi C (paratyphoid C) (*S. hirschfeldii*, Bergey's Manual) is very closely related serologically to some other members of group C, especially to *S. choleraesuis*. Culturally it differs from that organism in fermentation of trehalose, arabinose, and dulcitol, and by formation of hydrogen sulfide.

All three organisms may cause a clinical picture indistinguishable from that of typhoid fever, although the symptoms are apt to be milder. The organisms are present in the blood in the early stages of the infection, and later appear in the feces, and sometimes in the urine. Agglutinins develop, and the diagnosis may be made by agglutination and agglutinin absorption tests. The paratyphoid strains are more pathogenic for animals than is the typhoid bacillus. The development of antibodies in man and in animals is much less marked than that which occurs in typhoid infection. The disease is transmitted in the same ways as is typhoid fever, and some of the patients become chronic carriers.

The relative number of cases of paratyphoid fever as compared with typhoid varies greatly in different parts of the world. The C type has been found with relative frequency in parts of Asia, Africa, and southeastern Europe, but is almost unknown in the United States.

Immunization with vaccines is discussed in the section on typhoid fever.

Laboratory Diagnosis. The same methods are used as in typhoid fever. Precise identification of the organisms is sometimes difficult, especially in the case of paratyphoid B and paratyphoid C.

SALMONELLAS CAUSING GASTROENTERITIS

Salmonella infections also include those which are commonly referred to as "food poisoning" or "ptomaine poisoning." In this type of infection, food is indeed usually involved as an agent of transmission, but the term "ptomaine poisoning" is a misnomer. Almost any type of food may be implicated, depending on the opportunity of infection and the type of organism involved. The sources of infection are usually rodents, especially rats and mice, human carriers who handle food, eggs, and meat from diseased animals. Evidence indicates that gastroenteritis due to a salmonella is a true infection, and not caused by a preformed

Table 11

ANTIGENIC STRUCTURE OF SALMONELLA

Group	Type of Organism	Somatic O Antigen	Flagellar H Antigen	
			Phase 1	Phase 2
A	*S. paratyphi A* (paratyphoid A)	(I), I, II, XII	a	
B	*S. paratyphi B* (paratyphoid B) (*S. schottmuelleri*)	(I), IV, (V), XII	b	1, 2
	S. typhimurium (*S. aertrycke*)	(I), IV, (V), XII	i	1, 2, 3
	S. stanley	IV, V, XII	d	1, 2
	S. reading	IV, XII	e, h	1, 5
C	*S. choleraesuis* (*S. suipestifer*)	VI, VII	c	1, 5
	S. paratyphi C (paratyphoid C) (*S. hirschfeldii*)	VI, VII, (Vi)	c	1, 5
	S. thompson	VI, VII	k	1, 5
	S. newport	VI, VIII	e, h	1, 2, 3
D	*S. typhi*	IX, XII, (Vi)	d	
	S. enteritidis	(I), IX, XII	g, m	
	S. dublin	I, IX, XII	g, p	
	S. moscow	IX, XII	g, q	
	S. sendai	(I), IX, XII	a	1, 5
	S. panama	I, IX, XII	l, v	1, 5
	S. gallinarum (nonmotile)	IX, XII	..	

Note: ( ) = not always present.
Other groups beyond D have been described.

toxin. The symptoms comprise a more or less violent gastrointestinal disturbance, with sudden onset, nausea, vomiting, diarrhea, some fever, and prostration. Recovery is usually rapid, although occasionally a case terminates fatally. Sometimes the infection becomes generalized. There is usually an incubation period of a few hours. In the order of their frequency of occurrence the salmonellas usually involved are: *S. typhimurium, S. enteritidis,* and *S. choleraesuis.*

Laboratory Diagnosis. Diagnosis in cases of food poisoning due to salmonellas depends chiefly upon their isolation from the stools by plating methods. Occasionally they may be demonstrated in blood cultures. During convalescence agglutinins may be formed which can be tested against known strains of these bacteria. Cultures from the infected food should be made when possible.

Salmonella Typhimurium (*S. aertrycke, B. pestiscaviae*). This is the salmonella most frequently involved in food poisoning. It is primarily found in rodents, but also in birds (*B. psittacosis*). It is a common cause of epidemics among laboratory animals. It is a member of group B and very closely related to *S. paratyphi B* both culturally and serologically. Acid production in tartrate medium by *S. typhimurium* is of value in differentiation. Both *S. typhimurium* and *S. paratyphi B* are diphasic and in phase 1 they possess different flagellar antigens.

Salmonella Enteritidis (*B. enteritidis*) (*Gaertner, 1888*). This organism is often found in cattle. It has been isolated from cases of gastroenteritis caused by the ingestion of meat from diseased animals, or even of food contaminated by contact with the infected meat. The infection may be spread by the unclean handling

of food, by flies, or even by the contamination of food with the feces of mice or rats since it is found in these animals also. Rats and mice may recover from infection and become carriers of both *S. enteritidis* and *S. typhimurium.*

S. enteritidis closely resembles the paratyphoid B (*S. paratyphi B*) bacillus in its cultural reactions, but can be differentiated from it easily serologically as *S. enteritidis* is in group D.

Salmonella Choleraesuis (*S. suipestifer*). This organism was isolated by Salmon and Smith from swine with hog cholera, and was believed to be the etiologic agent in the cholera infection. It is now known, however, that the disease is caused by a filtrable virus and that, although this organism is constantly present and may be isolated from the blood, it is only a secondary invader. It has been reported as the cause of epidemics of food poisoning in man, although it is less common in that role than *S. typhimurium* and *S. enteritidis.* It has also been isolated from sporadic cases of a severe general infection in man, from spinal fluid in some cases of meningitis, from sputum in pneumonia, from joints, and abscesses. Identification of this organism requires precise agglutination tests. *S. choleraesuis* can also be differentiated by its inability to ferment arabinose, trehalose, and inositol.

Shigella or Dysentery Group

BACILLARY DYSENTERY

Acute bacillary dysentery is characterized by a severe inflammation of the mucous membrane of the large intestine and occasionally the lower part of the ileum, with the appearance of mucus, blood, and pus in the stools. The bacilli are present in the intestinal tract, and at times in the mesenteric lymph nodes, but they rarely invade the blood stream or appear in the urine.

All degrees of severity are encountered; some cases are so mild as to be practically symptomless, whereas others may be rapidly fatal. Atypical cases are common. Because of the occurrence of these and of subclinical infections many cases are not recognized. Recent intensive studies have revealed bacillary dysentery to be far more common than it was formerly thought to be.

The incubation period is usually two or three days, though it may be as short as 12 hours or as long as six days; the type of dysentery organism involved as well as the conditions of infection are the governing factors.

The onset may be gradual or sudden, and sometimes toxic systemic manifestations overshadow or obscure the intestinal symptoms. In the well-developed clinical picture abdominal cramps and severe tenesmus are accompanied by a violent diarrhea; as many as 50 to 100 stools may be passed in one day. These become small and bloody. Marked prostration and fever accompany this picture which may continue for several days. On the other hand there may be no symptoms other than a few loose stools; or in a mild case a watery diarrhea may last for several days.

The term "shigellosis" is now often used to designate all infections by members of the genus *Shigella.*

General Characteristics of the Bacteria of the Genus Shigella. The dysentery bacilli occur, as a rule, singly or in pairs, and do not form threads or filaments.

They are somewhat plumper than typhoid bacilli. In many cultural characteristics they resemble the typhoid bacillus, but can be distinguished from them by their lack of motility, certain biochemic reactions, and by agglutination (see table on inside of back cover). The shigellas differ from the salmonellas in three important respects: nonmotility, failure to produce hydrogen sulfide, and failure to form gas. Typhoid and dysentery bacilli show an identical reaction on Russell's double-sugar medium, but if Kligler's iron agar is used instead, the typhoid bacillus can be distinguished by the blackening of the medium due to hydrogen-sulfide production.

There are several species of *Shigella* that cause dysentery. These are discussed below.

Shigella Dysenteriae (*Shiga's bacillus, Shiga-Kruse Type, B. shigae*). This was the first of the dysentery bacilli to be described (Shiga, 1898) and causes the most severe type of infection. It is culturally and serologically homogeneous, and differs from other shigellas in that it produces a potent exotoxin. Evidence indicates that two toxins are produced: a soluble, heat-labile exotoxin (neurotoxin), chiefly affecting the nervous system, and a more thermostable enterotoxin, bound up with the cell protein. The neurotoxin produces a characteristic paralysis in monkeys, rabbits, and mice when given parenterally. Antitoxic sera are available for Shiga-type infections, and may be used in conjunction with other therapy.

Infections due to Shiga's bacillus occur most frequently in the Orient and in the "Middle East," but occasionally outbreaks or sporadic cases are reported in the United States. They probably occur, though infrequently, the world over.

S. dysenteriae does not ferment mannitol nor produce indol—two features of aid in its identification.

An organism which resembles the Shiga type in its inability to ferment mannitol but which produces indol and ferments rhamnose, is known as the Schmitz bacillus (*S. ambigua*). This organism does not produce a neurotoxin as does Shiga's bacillus, but only the enterotoxin common to all shigellas. Its distribution is similar to that of *S. dysenteriae*.

Several non-mannitol-fermenting members of this group, serologically distinct, have recently been described by Sachs (1943) as being common in India and Egypt. The name *S. arabinotarda* has been suggested (Christensen and Gowen, 1944) for these.

Shigella Paradysenteriae (*Flexner-Strong Type, Hiss Y, B. flexneri, B. dysenteriae, Flexner*). The organisms of this group are the commonest cause of bacillary dysentery in both temperate and warm countries. *S. paradysenteriae* does not produce a soluble exotoxin, though its cell substance is toxic.

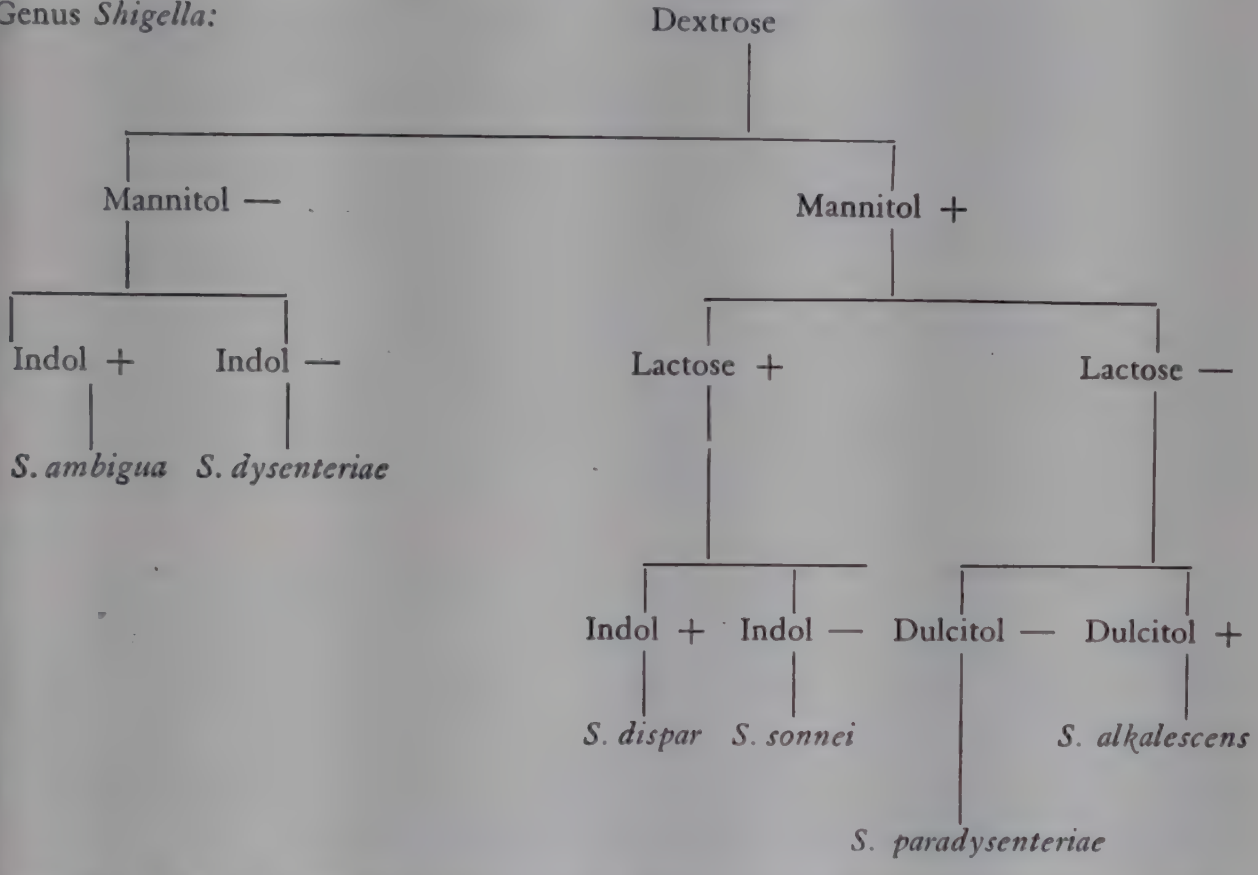
These so-called "Flexner" strains produce acid from mannitol and form indol. Although they are culturally and biochemically homogeneous, they are serologically heterogeneous, and a number of serologic types have been distinguished. Andrewes and Inman (1919) described five types which they designated V, W, X, Y, and Z. Boyd (1938) considered that X and Y are variants of others and reported three other types. He proposed the designation of six types by Roman numerals. Boyd's work has been essentially confirmed by Wheeler (1944) and extended by Weil and his co-workers (1943, 1944). This designation by Roman numerals has been widely adopted.

Since the shigellas are nonmotile, there are no flagellar antigens present, and they are, therefore, not diphasic. The dominating somatic factors are used as type-specific antigens

in serologic identification. This type-specificity seems to be determined by a polysaccharide hapten. Weil, Black, and Farsetta (1944) have proposed a classification of the shigella species (see Table 12). They list 14 types of *S. paradysenteriae* which they call *S. flexneri*. Some of these include strains which have been hitherto differently designated, such as type VI in which Boyd included the "Newcastle" strains which sometimes produce a small amount of gas. Many type III strains have been found to ferment rhamnose, and those of type XIII may give a positive trimethylamine-oxide reaction. As accurate typing of the shigellas becomes more widespread, data concerning their distribution will have greater significance.

A convenient scheme for provisional differentiation of the shigellas is shown in the following diagram (Naval Medical School Laboratory Guide, 1944).

Genus *Shigella:*



Hardy and Watt (1944) have found that in large-scale field studies the use of three sugars and an indol test is sufficient for a preliminary separation of species, as follows:

	Mannitol	*Xylose*	*Rhamnose*	*Indol*
S. paradysenteriae	+	—	∓	∓
S. sonnei	+	—	+	—
S. ambigua	—	—	+	+
S. alkalescens	+	+	—	+
S. dysenteriae	—	—	—	—
S. dispar	+	+	+	+

For other biochemic reactions see table on inside of back cover.

Shigella Sonnei (*Sonne, 1915*). *S. sonnei* has a widespread distribution and a high incidence. This organism ferments lactose and sucrose slowly, as well as mannitol, and does not produce indol. Rough (R) and smooth (S) variation is especially conspicuous in Sonne cultures, although this form of dissociation occurs freely in all of the shigellas.

Table 12

SEROLOGIC CLASSIFICATION OF THE GENUS SHIGELLA (AFTER WEIL, BLACK, AND FARSETTA)*

Present Name and Type	*Previous Designation*
Shigella dysenteriae	Shiga bacillus
S. ambigua	Schmitz bacillus
S. flexneri I	*S. paradysenteriae* V (A & I)†
S. flexneri II	*S. paradysenteriae* W (A & I)
S. flexneri III	*S. paradysenteriae* Z (A & I)
S. flexneri IV	Boyd type 103
S. flexneri V	Boyd type P 119
S. flexneri VI	Boyd 88—Newcastle-Manchester group
S. flexneri VII	*S. paradysenteriae* X (A & I)
S. flexneri VIII	*S. paradysenteriae* Y (A & I)
S. flexneri IX	Boyd type 170
S. flexneri X	Boyd type P 288
S. flexneri XI	Boyd type D 1
S. flexneri XII	Boyd type D 19
S. flexneri XIII	Boyd type P 143
S. flexneri XIV	Boyd type P 274
S. alkalescens	*B. alkalescens*
S. sonnei	Sonne's bacillus
S. ceylonensis	*B. dispar*
S. madampensis	*B. dispar*

*From Barnes (1944).

†A & I refers to Andrewes & Inman.

SHIGELLA CEYLONENSIS AND S. MADAMPENSIS. These shigellas have together been long known as *S. dispar*. They, too, ferment lactose and sucrose as well as mannitol, but, unlike Sonne, they produce indol.

SHIGELLA ALKALESCENS (*Andrewes, 1918*). *S. alkalescens* was for some time regarded as of doubtful pathogenicity, but accumulated evidence indicates that it, too, is probably responsible for shigellosis. Sometimes this organism produces H_2S.

Pathogenicity and Transmission. Shigellosis is primarily a disease of man. Epidemics due to the Flexner strains occur in laboratory colonies of monkeys, and spontaneous infections have been reported in dogs. When suspended in mucin and injected parenterally, a small number of virulent dysentery bacteria will kill mice in 12 to 48 hours. The Sonne type is more pathogenic for mice than are the other members of the group.

Although the route may be devious, transmission of shigellosis is from the infected intestinal tract of one individual to the mouth of another. Thus unrecognized cases and carriers are of great importance in this respect. Convalescents may carry the organisms for a number of months and some of them may show recurrent periods of excretion after intervals with negative findings. Some carriers have no history of the disease. Felsen (1945) believes that shigella carriers are usually sick carriers, and that this can be shown, in spite of the absence of symptoms, by careful sigmoidoscopic examinations. The carrier state with the shigelloses is not analogous to that with typhoid fever.

Laboratory Diagnosis. The appearance of the stools in acute bacillary dysentery is characteristic. There is usually an absence of fecal material, and the bulk of the stool is composed of clear or turbid, amber-colored, serous fluid in which float curled masses of white mucus flecked with bright-red blood. In amebic dysentery the typical stool contains fecal material and blood. It has been shown that the microscopic appearances of the stools of these two types of dysentery differ. The cell exudate in bacillary dysentery is abundant, and is composed largely of polymorphonuclear leukocytes. In amebic dysentery there are comparatively few cells in the exudate and mononuclear cells are more numerous than polymorphonuclears unless marked secondary infection is present (see p. 536). Warm-stage preparations may be searched for amebae.

During the first week the dysentery bacilli may be cultivated from the stools without difficulty. They are often present in large numbers, or even in practically pure culture in the first two or three days, but diminish rapidly in number as the fecal character of the stools returns. Isolation from chronic cases and from carriers is very difficult and uncertain.

The mucus is spread heavily on SS agar plates (see p. 354) on which the colonies are colorless. After identifying the organism by its cultural characteristics, final identification is made by agglutinating the organism obtained with specific antiserum. The use of absorbed sera is usually necessary to determine the precise type to which a Flexner strain belongs. The organism isolated should be tested later for agglutination with the patient's serum.

The development of agglutinins in the patient's blood occurs only after one or two weeks, and hence is of relatively little value in diagnosis. The agglutinin titer varies according to the type of shigella involved. With Flexner strains it may remain high for several years, and may also be found in non-shigella infections. The agglutination test on the patient's serum should be interpreted with care.

Vaccination. Vaccines of the Shiga bacillus made in the ordinary way are very toxic for man as well as for animals. Those of the paradysentery group may be used in small doses without detoxification. Their use in prophylaxis is still experimental, as is also the use of toxoids prepared from Shiga toxin.

Treatment. Several agents are now employed in the treatment of shigella infections. Of these the sulfonamides have the most general use. Some clinicians recommend the more insoluble members of this group—e.g., sulfaguanidine—which, when given by mouth, are absorbed and excreted more slowly, and remain in longer contact with the infecting organisms. In the United States, Hardy and Watt have had best success with the more soluble drugs—e.g., sulfadiazine. Different species of the shigellas vary in susceptibility to the sulfonamides; infections due to *S. sonnei* are especially unresponsive.

A polyvalent antiserum has been used in shigella infections for many years, but its value has been questioned. More recently an antitoxin for the Shiga type has been made available in a concentrated form, with increased potency. Results with its use on a large scale have not been reported, but by some it has been considered valuable in combating the intoxication caused by infection with this organism, especially when used in conjunction with the sulfonamides.

The question of treatment with specific bacteriophage has been reopened during World War II. Its use may be considered in the experimental stage.

Alkaligenes Faecalis (*B. faecalis alkaligenes*). This organism is a frequent inhabitant of the intestinal tract.

It does not ferment any of the sugars, and in milk cultures there is a progressive alkalinity, with the liberation of a little ammonia. It is strikingly aerobic.

This bacillus does not seem to have any effect on animals. It has been isolated from the blood of a few cases which resembled typhoid, and was agglutinated by their sera (1 : 50). It has been under suspicion in some cases of diarrhea in children.

Coliform Group (Escherichia and Aerobacter)

There are three genera in the tribe Eschericheae: *Escherichia, Aerobacter,* and *Klebsiella.* The relationship of *Klebsiella* to the other two genera is obvious, but its adaptation to the respiratory tract is responsible for many bacteriologists excluding it from a discussion of the coliform group. The genus *Klebsiella* has been described on p. 122. *Escherichia coli* and *Aerobacter aerogenes* are the type species of the other two genera.

Escherichia Coli (*B. coli*). This species includes a large number of types which differ in minor characteristics. The most important member of the group, *E. coli,* was isolated by Escherich in 1886 from the feces of infants. It is the common inhabitant of the intestinal tract of man and other animals, and its presence in water is an index of sewage contamination.

Morphology and Cultural Characteristics. *E. coli* is a Gram-negative, rod-shaped or oval organism with rounded ends, averaging about 0.5 × 1.0 to 3.0μ in size. It is motile, but less actively so than the typhoid bacillus. The degree of motility varies with different strains and with the same strain under different cultural conditions. It is most marked in young cultures (under 12 hours). The bacillus grows luxuriantly on all ordinary media and within a wide temperature range. In broth it produces a uniform turbidity, with, at times, a surface scum, and often a somewhat fetid odor. It acidifies and coagulates milk, and ferments many of the ordinary sugars, including glucose and lactose, with the production of acid and gas (see table on inside of back cover). Potato is discolored to a brownish hue. Gelatin is not liquefied. Indol is produced. On Endo plates the colonies are bright red and develop a metallic scum after about 36 hours. On other differential media they are a deep pink. Inoculated by streak and stab on Russell's tubes, the whole medium is acidified and gas develops in the butt.

Special tests are used to differentiate the colon bacillus from allied species. The most important of these are the production of indol from tryptophane, the degree of acidity produced in glucose broth (the methyl-red reaction), the formation of acetyl-methyl carbinol (Voges-Proskauer reaction), and the ability to use citrate as the sole source of carbon. These four tests, in the order named, are designated the "Imvic" reaction. With typical strains of *E. coli* the Voges-Proskauer reaction is negative, and reaction to the methyl-red test is positive. The colon bacilli are unable to utilize citrate as a source of carbon, as does *A. aerogenes,* and do not grow in the synthetic citrate medium. Different strains of *E. coli* vary in many respects. Some fail to produce gas, some ferment sucrose (*E. communior*), and some pathogenic strains are apt to have capsules, hemolyze red blood cells, and ferment lactose very slowly.

Pathogenicity. The colon bacilli appear in the intestinal tract shortly after birth, and continue to be present in large numbers throughout life. It is probable that they exert some protective action in the intestinal tract, but they are capable of producing a variety

of infections elsewhere in the body. They are particularly prone to cause urinary tract infections, and in young children a colon bacillus pyelitis is a frequent cause of fever. They often cause infections of the gall-bladder and biliary passages, peritonitis from perforation of the gastrointestinal tract (often in association with other organisms), and suppurative lesions in any part of the body. Colon bacillus infections are more common in the tropics than in temperate climates, and cases of prolonged fever may be due to a colon bacillus bacteremia. Owing to the tendency of these bacilli to postmortem or agonal invasion of the blood stream, a positive culture at this time has little or no significance.

Aerobacter Aerogenes (*B. lactis aerogenes*). This organism is found in the intestinal tract of man and other animals. It seems able, too, to lead a saprophytic existence in nature and may be found on grains and in soils. Thus its sanitary significance in milk or water is different from that of *E. coli*. Like *E. communior* it produces gas in glucose, lactose, and saccharose broth, but does not ferment dulcitol. It is, as a rule, distinctly nonmotile, and often seems to be surrounded by capsular material. The colonies are large and very viscid. Except for its greater fermentative activities it might be confused with the Friedländer bacillus. Unlike the colon bacillus it gives a positive Voges-Proskauer reaction, and is methyl red-negative. It frequently grows in the synthetic citrate medium. Gelatin is not liquefied. It has been reported as a cause of cystitis.

The Paracolon Bacteria. These organisms are characterized by late lactose fermentation (5 to 14 days), and some strains fail to produce gas. Some contain antigens found in the Salmonella group. Occasional strains are somewhat pathogenic.

Detection of the Coliform Bacteria in Water Supplies. The presence of intestinal bacteria in water, whether it be from streams, wells, or springs, is taken as an indication of contamination by sewage. Although some countries recognize *Clostridium perfringens* or enterococci as indicators of such contamination, *E. coli* is the organism most generally adopted for this purpose. The detection of these bacilli which are so abundant in the intestines, and so active biochemically, is much more practical than attempting to find such occasional pathogens as typhoid bacilli.

There are three steps in a completed test for the presence of the coliform bacteria.

1. Presumptive Test. A series of lactose-broth fermentation tubes is inoculated with graduated quantities of the water (10 ml., 1.0 ml., 0.1 ml., etc.). The quantity of the medium must be at least twice that of the water. The tubes are incubated at 37° C. for 48 hours. They are examined at both 24 and 48 hours and gas formation recorded. The formation of gas occupying more than 10 per cent of the inverted vial or closed arm of tube within 24 hours constitutes a positive reaction to the presumptive test. If the gas formed in 24 hours is less than 10 per cent, or if none is seen within the 48 hours, the reaction is doubtful and must be confirmed. The absence of gas after 48 hours constitutes a negative reaction.

2. Partially Confirmed Test. From the tubes showing gas the one which contains the smallest amount of water is chosen for making two or more plates on a differential agar medium (Endo, eosin-methylene blue, MacConkey's). The plates are incubated at 37° C. for from 18 to 24 hours. If typical colonies develop in this period, the reaction to the partially confirmed test may be considered positive. If, however, no typical colonies appear the reaction is not definitely negative. The test must be completed.

3. Completed Test. Two or more of the most typical colonies are transferred to an agar slant and to a lactose-broth fermentation tube. If no typical colonies appear within 24 hours the tubes are incubated for another 24 hours. Then two or more of the colonies most likely to belong to the group, whether typical or not, are transferred to agar slants and to lactose-broth fermentation tubes.

The inoculated tubes are incubated until gas forms or for 48 hours. The agar slants are incubated for 18 to 24 hours. Then at least one slant culture, corresponding to a tube which shows gas formation, is examined microscopically.

The formation of gas in lactose broth and the demonstration of motile Gram-negative,

non-spore-bearing bacilli in the agar slants are complete proof of the presence of a member of the coliform group.

The absence of gas formation, or failure to demonstrate such bacilli, constitutes a negative reaction.

When examining potable water at least five tubes should be inoculated with 10 ml. of water. The presence of organisms of the coliform group in more than one of the 10-ml. samples may be regarded as evidence of contamination.

The *E. coli* and *Aerobacter aerogenes* groups may be further differentiated by the Imvic group of tests. More detailed description of the bacteriologic and chemical examination of water both quantitatively and qualitatively, may be found in the *Standard Methods of Water Analysis* published by the American Public Health Association, Ninth Edition (1946).

Gelatin-liquefying Fecal Bacteria

Aerobacter Cloacae (*B. cloacae*) (*Isolated First from Sewage by Jordan*). *A. cloacae* is, as a rule, a liquefier of gelatin, although not as active as the proteus group. In its motility and in its reactions with milk and the sugars it resembles the colon bacillus. The Voges-Proskauer reaction is positive, the methyl-red reaction negative, and, like the *A. aerogenes,* it gives a positive reaction to the citrate-utilization test.

Proteus Vulgaris. This organism is often encountered in plates made from water contaminated with sewage, and in routine stool examinations for pathogenic enteric microörganisms. Certain strains are agglutinated by the serum of patients with typhus fever and other rickettsial infections.

Morphology and Cultural Characteristics. The bacillus is very motile, long and slender, tending to form filaments. It is Gram-negative. The spreading growth on solid media is characteristic of the group. It is perhaps the commonest of the "spreaders" encountered on Petri dishes in enteric bacteriology. From the central colony irregular, translucent streamers spread out and coalesce until the whole surface of the medium is covered. Glucose, maltose, and generally saccharose are fermented, but not lactose. The reaction on Russell's double-sugar medium is, therefore, identical with that of the salmonellas. The use of a triple-sugar medium such as T.S.I., or Krumweide's, avoids this confusion, as also does Kligler's iron agar since the proteus organisms produce hydrogen sulfide. A valuable means of identifying the proteus organisms is found in their ability to decompose urea (Rustigan and Stuart, 1941) (see p. 351). Milk is coagulated and alkalinized. Later the indicator is reduced, the clot digested, and the medium becomes a dirty yellowish-brown fluid. Indol is rarely produced. The cultures generally have a putrefactive odor. Gelatin is rapidly liquefied, and some strains liquefy coagulated blood serum.

Pathogenicity. *Proteus vulgaris* is common in putrefying organic material. It has been reported as the cause of acute food poisoning, and has been found in the blood in these cases. It may cause various suppurative processes, such as otitis media, peritonitis, cystitis, and pyelitis, usually in association with other organisms.

Variation. Nonmotile forms of *Proteus vulgaris* occur which grow in round, thick colonies without the characteristic spreading. Weil and Felix applied the term O (ohne Hauch) to their strains of this variety in contrast to the motile spreading types which they designated as H (Hauch). Since their original work, the letter H has come to be applied to the flagellar antigen and antibodies of various motile bacteria, and the letter O to the somatic antigens and antibodies of the nonmotile forms.

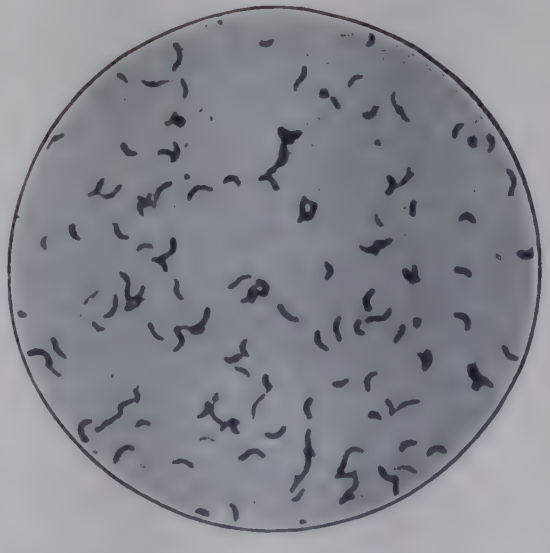
Weil-Felix Reaction. Weil and Felix isolated from the urine of three typhus fever patients two strains of *Proteus vulgaris* which they designated as X_2 and X_{19}. These strains are nonmotile and nonspreading (ohne Hauch), and, therefore, represent the O form. These strains are agglutinated by the sera of patients with typhus fever and other rickettsial diseases, although they are neither the causative agents nor secondary invaders. The reaction is, therefore, heterologous and nonspecific, but it is an almost constant phenomenon, is not present in other conditions, and is of great practical value in diagnosis. The test is described in detail in the section on typhus fever.

Proteus Morgani (*Salmonella morgani, Morgan's Bacillus, Morgan's No. 1*). This bacillus was isolated by Morgan (1906) from the stools of infants with summer diarrhea. It has been classified with one group after another. It does not liquefy gelatin, and produces indol. It is now placed in the genus *Proteus* instead of *Salmonella*. It seems to play a role in some diarrheas of infants.

Cholera Vibrios

Vibrio Comma (*Vibrio cholerae asiaticae, Spirillum cholerae asiaticae*) (*Koch, 1884*). This organism is the cause of Asiatic cholera.

Morphology. Typically, the organism is a small, slightly curved rod, 0.4 to 0.6 × 1.5 to 3.0μ. It may occur in S shapes, owing to the end-to-end attachment of a pair of organisms; in cultures long threads showing a somewhat spiral appearance may be seen. In smears made from bits of mucus and cellular debris in the feces, the bacteria often resemble fish swimming parallel to one another in a stream. After artificial cultivation, and occasionally in freshly isolated cultures, rod forms, coccoid, and club-shaped involution forms are frequent. Ohno found that their development depends in part upon the reaction of the medium, and suggested that transfers be made on media of varying pH to obtain the characteristic vibrio morphology. There is a single, long, terminal flagellum which imparts to the organism a very active "scintillating" or "darting" motility. *Vibrio comma* stains easily by ordinary methods and is Gram-negative. Commonly *Vibrio comma* is much less curved than its name indicates. It was named by Robert Koch and the old German script comma is nearly straight.



Cholera vibrios. (Kolle and Wassermann.)

Cultural Characteristics. The *Vibrio comma* is strictly aerobic and grows readily upon ordinary culture media. The optimum reaction is pH 8.0 to 9.0. Growth is inhibited by a moderate acidity, but will occur on media sufficiently alkaline to inhibit other species of bacteria. This tolerance for alkalinity facilitates the isolation of these organisms from the feces by special media. On agar the colonies are translucent, bluish gray, resembling somewhat those of the typhoid bacillus. On gelatin plates they are more characteristic, and appear after 24 hours as small, highly refractile, whitish colonies which, under the low power, have a granular center with spinose margins, and a surrounding zone of liquefaction. In gelatin stabs incubated at room temperature a turnip-shaped area of liquefaction appears at the top of the puncture—the air-bubble appearance. Coagulated

blood serum is liquefied. Milk is (usually) not acidified nor coagulated. On alkaline potato the growth is whitish and later changes to a brownish-yellow or pinkish color. In broth or in Dunham's peptone solution growth is rapid and luxuriant, especially at the surface, and a pellicle is formed. Pellicle formation is correlated with roughness or smoothness of colonies. In the latter medium indol is produced, and the nitrates are reduced to nitrites. The addition of a few drops of concentrated sulfuric acid alone will bring out a red color due to the formation of nitroso-indol—the cholera-red reaction (see p. 358). The ability to ferment carbohydrates varies somewhat with different strains. Three sugars are the basis of a useful classification into six groups, as described by Heiberg (1935). All of the true cholera vibrios and also some noncholera varieties are found in group I. Strains of this group ferment mannose and saccharose, and fail to ferment arabinose. Since many kinds of vibrios are found in nature, especially in water and feces, such a simple means of ruling out nonpathogenic strains is of great practical importance. Strains that are found not to fall into group I do not have to be examined serologically.

The typical cholera vibrio does not produce hemolysis on blood media, although after several days growth there may be some chemical alteration or digestion of the medium around the colony which simulates a zone of hemolysis. Cooked blood medium is cleared in the same way. If a filtrate from a broth culture is added to a suspension of red blood cells, no hemolysis occurs. An exception to this rule is the El Tor vibrio which was isolated from cases of fatal diarrhea in pilgrims at El Tor. This organism, which also falls into group I, is actively hemolytic, yet is agglutinated by cholera immune serum.

Serologic Relationships. Most vibrios have a common H (flagellar) antigen whereas the O (somatic) antigens are specific. Gardner and Venkatraman (1935), using antisera produced with heat-stable antigens, found that most vibrios may be placed in one of six groups on the basis of the specific agglutinability of the O antigen. True cholera vibrios, and some of the El Tor strains, fall into group I of this classification system. Serologic identification of the cholera vibrio depends, therefore, on the use of a specific O antiserum.

Three types of cholera vibrios have been described within group I by means of serologic reactions: Inaba, Ogawa, and Hikojima. Since there are epidemiologic and immunologic differences among these types it becomes important to determine the type of vibrio. Absorbed antisera used in agglutination reactions will determine the type. Inaba and Ogawa types are most common in India and China, while the Hikojima type has been described as causing outbreaks in Japan and occasionally on the coast of China. Burrows (1946) has found three antigens which he has designated as A, B, and C. On the basis of the distribution of these among the cholera vibrios he has proposed a new classification.

The six fermentation groups of Heiberg and the six serologic groups are not identical. True cholera vibrios are contained, however, in group I of each classification.

The cholera vibrio is readily dissociated, and a number of variant strains have been produced by various methods of cultivation. According to Linton et al., these changes depend primarily upon a loss or chemical alteration of the specific carbohydrate.

Toxic Products. No true soluble exotoxin has ever been demonstrated in cholera vibrios. The endotoxins of the cells are especially toxic and a profound toxemia seems to occur in human cases.

Pathogenicity. Feeding, or subcutaneous injection of the cholera vibrio does not usually cause infection in adult animals. Koch, however, produced the disease in guinea pigs by injecting the organisms together with alkali into the stomach, and giving them opiates to inhibit intestinal peristalsis. Intraperitoneal injection usually gives rise to a fatal peritonitis. However, when the organisms are injected into an immunized guinea pig, or when a small amount of immune serum is simultaneously injected, bacteriolysis takes place (*Pfeiffer's phenomenon*). If material is removed from the peritoneal cavity with a pipet, at intervals of from 10 to 60 minutes, the cholera vibrios will be found to have lost their motility

and to have become granular and degenerated. Other vibrios are unchanged. The reaction may be demonstrated in a pipet if fresh serum is used. Mice are susceptible if the vibrios, suspended in mucin, are injected intraperitoneally. Griffitts (1942) has used this method for the evaluation of cholera vaccines. Agglutinating and bacteriolytic sera of high titer can be prepared by the immunization of animals.

Pigeons are almost insusceptible to the true cholera vibrio but are readily infected by a closely allied species, *V. metchnikovi*, which is not pathogenic for man. It is more pathogenic for guinea pigs than is the *V. comma*.

Transmission. Cholera is endemic in India, and practically every pandemic has been traced to this area. Another endemic focus is believed to exist in China. The disease is spread by contamination of water supplies or food by feces from a patient or from a carrier. The organism may live for weeks in stagnant water, and great explosive outbreaks have occurred from polluted water. Ordinarily the vibrios disappear rapidly from the feces (within 7 to 14 days), but Grieg has shown that the biliary tract is often infected, and that they may persist here for a long period of time. Virulent organisms have been demonstrated also in the feces of healthy individuals who have been in contact with cholera cases. On the other hand, epidemiologic observations indicate that even typical agglutinable vibrios from the feces may not be virulent, and the possibility of the dissemination of the disease by chronic carriers has been questioned.

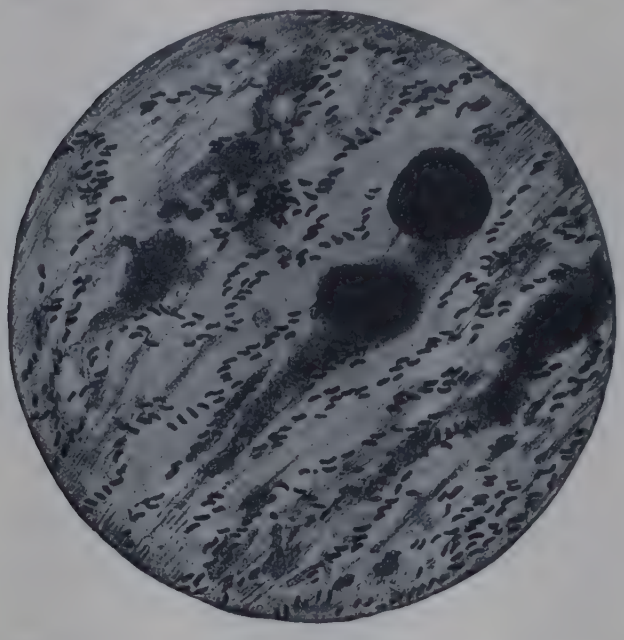
Cholera in Man. The onset is abrupt after an incubation period of from two to five days. A profuse diarrhea occurs, usually without colic or tenesmus. The stools soon lose their fecal character and consist of a watery, slightly opaque fluid containing flocculi of desquamated epithelium and mucus (the characteristic rice-water stools). Similar material is vomited. At this stage muscle cramps are frequent, probably associated with the great loss in chlorides caused by the diarrhea and vomiting. There is great toxemia which probably depends in part upon the absorption of the endotoxin. The dehydration is intense, and there is anuria, low systolic pressure, and cyanosis. The subcutaneous tissues are so shrunken that the body has a cadaveric appearance, and the hands look shriveled, the typical so-called "washerwoman's hands." At this stage the blood becomes greatly concentrated, the red blood cells frequently reaching 7 or 8 millions, with a corresponding increase in the leukocytes. The proteins of the blood are concentrated, and the specific gravity is raised. As a result of the anuria and the increased tissue destruction, the nonprotein nitrogen of the blood may reach high figures. Sellards found that there is also an acidosis (due to loss of base through the intestinal tract); he witnessed great improvement in patients who were given intravenous injections of sodium bicarbonate solution. Equally good results follow large and repeated injections of hypertonic salt solution, to which glucose may be added. If anuria is relieved, the kidney seems to be able to restore the acid-base equilibrium. In untreated patients the fatality rate is over 50 per cent.

The vibrios disappear rapidly after recovery, and chronic carriers probably do not exist.

Laboratory Diagnosis. During the acute stage of the disease the vibrios can be demonstrated in large numbers in smears from the rice-water stools and by cultures of the stools. They do not penetrate into the submucosa, and are seldom if ever found in the blood stream. Exceptionally they have been isolated from the urine, and Grieg has found them in the lungs in convalescents who developed

pneumonia. He believes that they are carried to these areas by the lymphatics. In the later stages the diagnosis may be made by agglutination tests.

FECES. The organisms may be recognized in smears from a fleck of mucus stained with a 1 : 10 dilution of carbolfuchsin. In such preparations they frequently show the "fish in a stream" appearance. In feces the vibrios often appear to be in "pure culture."



Cholera vibrios—"fish in the stream appearance." (After Jochmann from Mayer.)

For cultivation the methods of Linton (1945) are recommended and these are quoted extensively below. When the vibrios are abundant and the material can be handled at once, stools may be plated out directly. If it is necessary to preserve stool material or to transport it, the buffered boric acid-potassium chloride mixture described by Venkatraman and Ramakrishnan (1941) is recommended. Successful isolation up to 92 days has been reported when this preserving fluid was used. (*Ind. J. M. Res.*, 1941.)

When the stool specimens arrive in the laboratory they are placed in a bismuth-sulfite enrichment medium (Wilson and Reilly, 1940), and incubated at 37° C. for 24 to 48 hours and then plated out. A tube of alkaline (pH = 8 to 9) peptone solution may also serve as an enrichment medium if incubated for only a few hours. Plating can be done on Aronson's medium (p. 357), or on plain nutrient agar (p. 341). The SS (Salmonella-Shigella) medium (p. 354) may also be used. On Aronson's medium, vibrio colonies are red and colonies of the colon bacilli are colorless and larger. Plates of Aronson's medium should be not over three days old and should be kept in a cold, dark place.

Colonies (at least 10) may be picked and tested on a slide with an O antiserum. If agglutination occurs, more extensive tests may be run. The colonies may be picked and these cultures put into three sugars; those giving the reaction of Heiberg's group I (mannose and saccharose = +, arabinose = 0) should be studied serologically and for other characteristic reactions.

Final identification of the organism is made by agglutinating it with a cholera immune serum, preferably one prepared with the O antigen only. The demonstration of Pfeiffer's phenomenon with immune serum is the surest proof that the suspected organism is a true cholera vibrio.

SEROLOGIC TESTS. H + O agglutinating sera are prepared by intravenous injection into rabbits of living suspensions of vibrios in increasing doses at five-day intervals. Three to five injections will usually give a good titer.

For O antisera the suspensions of vibrios are heated in a boiling water bath for one to two hours to destroy the H components. Intravenous injection of rabbits is done as above. A high titer is less often obtained with this antigen. According to Linton, sera with a lower titer than 1 : 500 should not be used. Another means of obtaining an O antigen is by successive replating and picking of translucent colonies of a culture. These O sera are sufficient for most diagnostic work. If differentiation between the types within the group is indicated, the sera can be rendered specific for these by absorption with the heterologous organisms.

Agglutination tests with these sera are set up as usual, but are incubated four hours at 52° C., and left at room temperature until the following day before recording results.

Agglutinins appear in the patient's serum about the fourth day and reach a maximum in about two weeks. They may be demonstrated by agglutination tests with a known cholera strain. The titer is variable, from 1 : 100 to 1 : 1000 or higher. Pfeiffer's phenomenon may be obtained with the serum when the agglutination is not clear-cut.

WATER. For isolation of the vibrios from water, samples are collected in sterile quart bottles, salt or sea-salt mixture is added to make a 1 to 2 per cent solution, and the pH adjusted to 9.2 with sodium hydroxide. When such samples reach the laboratory they are filtered through Seitz pads and the pad placed in the enrichment medium. From this point on the procedure is the same as for stools.

IMMUNIZATION. An attack of cholera confers a lasting immunity. Second cases have not been definitely proved. Prophylactic vaccination was first attempted by Ferran in 1885 and later by Haffkine with live cultures. Kolle first introduced the use of killed cultures, which are employed exclusively at present. These vaccines may be prepared by the same methods as typhoid vaccine. Two doses of 4000 and 8000 million vibrios are given with an interval of about a week. Castellani combines them with the triple typhoid vaccine. Reports have shown that vaccination has been of considerable value during epidemics, both in decreasing the incidence of infection and in reducing the mortality.

In several large series the morbidity among the inoculated has been reduced to from one-quarter to one-tenth of that among those not inoculated. The mortality among those attacked was also reduced, but to a much less extent (about one-half in some series).

Paracholera Vibrios. Various other organisms resembling the true cholera vibrio have been isolated from cholera-like conditions. They differ in minor characteristics and are not agglutinated by an anticholera O serum. They have been designated the paracholera vibrios. Five groups have been differentiated, based on differences in their O antigens.

Nonmotile, nonliquefying vibrios have been described. Some are also phosphorescent.

Chromogenic Bacilli

Pseudomonas Aeruginosa (*B. pyocyaneus*) (*Gessard, 1882*). There are about thirty species of the genus Pseudomonas, most of which are fluorescent, motile, and gelatin-liquefying. *P. aeruginosa* is the species of medical importance. This organism is frequently termed the bacillus of green or blue pus. It is widely distributed in water and air, on the skin, and in the upper respiratory tract, and is often isolated from feces. It is associated sometimes with other pyogenic organisms in abdominal abscesses. It is probably more pathogenic than has been supposed, and is sometimes the sole cause of cystitis, otitis media, mastoiditis, meningitis, enteritis in children, and, occasionally, even of septicemia.

MORPHOLOGY AND CULTURAL CHARACTERISTICS. This bacillus is small, slender (0.5 × 1.0 to 2.5μ), motile, and Gram-negative. It grows readily at room or body temperature. Growth on agar is abundant, moist, and greenish, and a bright-green color is diffused through the agar. Gelatin is rapidly liquefied. On potato the colonies are a deep olive-green to dirty brown. Blood serum is digested, and the pitted surface shows a reddish-brown color. No gas is produced from any of the carbohydrates, and acid is formed only from glucose. No indol is produced.

In the presence of oxygen, pigments are formed which have been separated into two main types: (1) pyocyanin, a blue-green pigment, soluble in both water and chloroform, and (2) fluorescin, a yellowish-green, fluorescent pigment, soluble in water but not in chloroform. A red pigment, pyorubin, has also been described.

A large number of variants have been described—R forms, nonmotile, and nonpigment-producing strains; and an encapsulated variety has been reported.

TOXINS. In addition to an endotoxin, the pyocyaneus bacillus produces a soluble toxin

similar to the diphtheria and the tetanus toxin, but different in that it will withstand a temperature of 100° C. for a short time and is much less potent than either the diphtheria or the tetanus toxin.

The fact that the union between toxin and antitoxin is only of a binding, neutralizing nature is shown by taking a mixture of pyocyaneus toxin and antitoxin, which is innocuous, and heating it to 100° C. This destroys the antitoxin, but does not injure the toxin, which retains its original toxicity. On the other hand the toxins of diphtheria and tetanus are less stable than the corresponding antitoxins, and, therefore, heating would destroy the toxins first.

The *B. fluorescens liquefaciens* of water seems to be simply a strain of *P. aeruginosa.*

Serratia Marcescens (*B. prodigiosus*). This is a very small, motile coccobacillus. It is Gram-negative. The colonies on agar or other solid media show a rich red pigment which develops only at room temperature. *S. marcescens* is frequently found on foodstuff, especially bread, where it has been mistaken for blood. It liquefies gelatin rapidly and produces a diffuse turbidity in broth. It is probable that *S. indica* and *S. kiliensis* are strains of *S. marcescens.*

On account of its size and visibility it is often used to test for leaks in filters. It should be held back by Berkefeld V filters.

Chromobacterium Violaceum (*B. violaceus*). This bacterium, a violet chromogen, has been described under many names. It has been found in water and soil. At times it may be definitely pathogenic. It has frequently been found in abscesses and several cases of fatal infection have been reported. It is said on good authority that such cases are really due to *C. ianthinum,* and that *C. violaceum* does not grow at 37° C.

This Gram-negative, motile organism produces a deep-violet pigment, soluble in alcohol but not in chloroform. Gelatin is liquefied and indol is not produced.

Non-spore-forming Anaerobes

This miscellaneous group is composed of a number of organisms which are probably not related. They are little known and often overlooked because they are anaerobic, non-spore-forming, and usually Gram-negative. They are found in the mouth, intestinal tract, and genital tract, and are frequently associated with other organisms in suppurative processes. Wherever they have been looked for they have been often found. Dack (1940) found them in 200 of 5180 specimens submitted in the Department of Surgery of the University of Chicago. Many of them belong to the two genera *Fusobacterium* and *Bacteroides.* Members of the genus *Fusobacterium* (*F. plauti-vincenti*) are found in the mouth and may be associated with Vincent's angina. The classification of these organisms is discussed in connection with Vincent's angina on p. 155. *Dialister pneumosintes,* found in the nasopharynx, is discussed on p. 106.

The members of the genus *Bacteroides* are neither rare nor difficult to find. They are, however, often overlooked since they are anaerobic, usually grow slowly, and often require enriched media. They have been found in the intestinal tract of healthy and diseased persons and animals, and have been encountered also in a variety of human infections such as appendicitis, peritonitis, mastoiditis, and abscesses of lung, liver, and urinary tract. Of the many species that have been described those most frequently met in human infections are: (1) *Bacteroides fragilis,* a delicate organism, rather difficult to grow, and short-lived; (2) *Bacteroides melanogenicus,* also delicate, which produces a coal-black pigment on blood agar by the fourth or fifth day; (3) *Bacteroides funduliformis,* a little easier to cultivate and slightly more active biochemically; and (4) *Bacteroides serpens* which produces gas as well as acid in a number of carbohydrates, produces indol, and blackens brain medium.

Streptobacillus Moniliformis

The classification of *Streptobacillus moniliformis* is uncertain, but by common usage this term has been tentatively accepted. Initially isolated by Schottmueller (1914) in Germany and by Blake (1916) in the United States from cases of rat-bite fever, the infection's rodent source was demonstrated by Tunnicliff (1916) who found it in the lungs of rats. A milk-borne outbreak in Haverhill, Massachusetts, (Parker and Hudson, 1926) due to this organism was designated as Haverhill fever and the organism at that time termed *Haverhillia multiformis*. It has also been called *Actinomyces muris ratti*.

The organism is very pleomorphic in cultures, with filaments that are fragmented into bacillary and coccoid forms. It is Gram-negative.

Staining is difficult. Wayson's stain (p. 366) is recommended. After fixation the film should be stained for 20 seconds and then flooded with alcohol. Then the slide should be dried immediately with a stream of air. Viable organisms are stained blue, and dead ones pink. A relation to the pleuropneumonia group of organisms has been suggested.

The medium of choice for isolation is a tryptose phosphate broth containing 20 per cent of horse serum, or a dextrose starch broth similarly enriched (p. 346). The most favorable pH is about 7.6. Solid media may be made, if preferred, by adding agar to these broths.

For routine isolation purposes it is necessary that serial transfers in the above fluid media be made daily for about a week. The growth is characteristic with fluffy, white colonies of about 1 to 2 mm. settling at the bottom of the tube. The organism will grow on Loeffler's serum and on egg-yolk medium. Daily transfer is unnecessary after the organism is established on solid media. Cultures will live a week at 4° C., and will remain viable indefinitely when dried from a frozen state.

Rat-bite Fever Due to Streptobacillus Moniliformis. Rat-bite fever is a term used to describe a specific clinical disease caused by the bite of a rat. The disease is due to either *Spirillum minus* or *Streptobacillus moniliformis*. Although certain differences in the clinical aspects of the two infections have been reported, it is only by demonstration of the causative organism that diagnosis can be accurately made. The infection caused by *Sp. minus* is discussed in another chapter (p. 160).

The incubation period of rat-bite fever due to *Streptobacillus moniliformis* is shorter than that due to *Sp. minus*. The onset of illness is usually sudden with symptoms of a generalized infection. The fever tends to be intermittent, or periodic. A scarlatiniform or morbilliform rash usually appears at the onset of the second bout of fever. Arthritis involving one or more joints also occurs at this time; this arthritis is transient and migratory. The organism can be isolated from joint fluid as well as from the blood. A false-positive serologic reaction for syphilis may be obtained. The course of illness is frequently prolonged, the average case lasting about a month.

For demonstrating the etiologic agent, methods should be employed that are suitable for either *Streptobacillus moniliformis* or *Sp. minus*. Larson has suggested that the following procedure be employed:

1. Defibrinated blood is taken at the onset and at the height of fever.
2. (a) Liquid medium is inoculated with 1 to 2 ml.; (b) rats and mice are inoculated

intraperitoneally, and mice are inoculated in the footpads. (These animals should be examined prior to inoculation for *Sp. minus.*)

3. Cultures on artificial media should be observed as previously stated.

4. The blood and peritoneal fluid of inoculated animals should be examined daily under dark-field illumination for at least two weeks. Organisms demonstrable within 24 to 48 hours have no significance. If *Streptobacillus moniliformis* is present, mice may die in 48 hours with no gross lesions. If death occurs in a week the spleen is large, and necrotic foci may be found in the spleen and liver. Organisms may be isolated from these organs. Mice injected in the footpads will develop arthritis in a week, and the organism may be cultivated from the fluid. *Streptobacillus moniliformis* has never been visually demonstrated in the tissues of the affected areas.

Penicillin is the treatment of choice.

Pleuropneumonia Group of Organisms

For many years these peculiar microörganisms were considered to be filtrable viruses, and were long cited as the only viruses to be cultivable. During recent years they have been recognized as a distinct group of filtrable parasitic and saprophytic organisms, different from viruses, ordinary bacteria, or rickettsiae.

These organisms will grow in cell-free media as pleomorphic rings, globules, granules, or filaments. They are considered Gram-negative, but stain with difficulty with the usual bacterial stains. Methods used for protozoa are said to be more successful. They are filtrable, and will grow aerobically or anaerobically, preferring a pH of 7.8 to 8.0. They will form colonies on suitable media. Isolation is best accomplished in an agar medium containing 30 per cent serum or ascitic fluid. The organisms will produce acid in some sugars. Cultures will live for weeks or months if sealed and refrigerated.

Classification of these organisms is very unsettled. They were, at one time, placed in the family Actinomycetaceae. Sabin (1941) has proposed a classification into two families and two genera. Dienes (1945) has suggested that they be placed with the Pasteurellas. They have been isolated from a number of bacterial cultures. Kleineberger (1942) found them in all strains of *Streptobacillus moniliformis* which she examined. A relationship has been suggested though Kleineberger considers the pleuropneumonia group to be distinct from *Streptobacillus moniliformis.*

Organisms of the pleuropneumonia group are the etiologic agent of pleuropneumonia of cattle and agalactia of sheep and goats. They have been found in canine distemper and in mice and rats, although no role in the cause of disease has been proved. Dienes (1940) has reported finding such organisms in man.

Donovan's Bodies

Donovania Granulomatis (*Anderson, DeMonbreun, and Goodpasture, 1945*). This organism was first described by Donovan (1905) as occurring in the lesions of granuloma inguinale as a coccoid or short, rod-shaped body, surrounded by a thick halo or capsule, found within a mononuclear phagocyte. The organisms are Gram-negative but stain well with Giemsa's stain. Similar unencapsulated bodies are found outside the cells in the lesions of granuloma inguinale and may show

bizarre variations in structure. They were at first regarded as protozoa, and commonly have been known as Donovan's bodies.

Donovania granulomatis cannot be grown on ordinary media nor on the chorioallantoic membrane of chick embryos, and the laboratory animals thus far tested, including rhesus monkeys, have been insusceptible to inoculation. Anderson et al. obtained cultures in the yolk sac of developing chick embryos by inoculating uncontaminated material from the lesions of three persons with granuloma inguinale. The organisms grew abundantly in the yolk and in the cells of the yolk sac but did not invade the other tissues of the embryo. Anderson et al. also cultivated the organisms *in vitro* in yolk aspirated from developing embryos (not in that from unfertilized eggs). In their growth requirements the organisms seem to be intermediate between the bacteria and the viruses in requiring substances liberated by growing cells, although they are not strictly obligate intracellular parasites. Anderson et al. regarded them as bacteria.

Using material from chick-embryo cultures as antigen, Anderson et al. obtained positive cutaneous allergic reactions in patients with granuloma inguinale, and positive precipitin and complement-fixation reactions with serum from such patients. Although these reactions have not been studied sufficiently to warrant their use as diagnostic tests, they appeared to be highly specific and to furnish strong evidence that *Donovania granulomatis* is the causative agent of granuloma inguinale. Production of the disease in man by inoculation of cultures has not been reported.

Granuloma Inguinale. Granuloma inguinale is a venereal infection, largely limited to negroes, which is widespread in the tropics including the West Indies, and is not uncommon in the southern portion of the United States. The lesions are chiefly limited to the genital regions, starting as small vesicles which spread over the skin and mucous membranes, become excoriated, and produce chronic, foul ulcers which are often covered by exuberant granulation tissue. They rarely involve the deep tissues, however, and have relatively little effect on the general health. Diagnosis is made by demonstrating Donovan's bodies within the cells in scrapings from the lesions.

CHAPTER 6

Spirochetes

Classification

It is still unsettled whether the spirochetes should be considered as protozoa, as suggested by Schaudinn 30 years ago, or whether they are more closely related to the bacteria as was originally believed. At present the latter view is more generally held, and Bergey, in the last *Manual of Determinative Bacteriology,* places them in the class Schizomycetes, order Spirochaetales.

Noguchi first proposed a classification of the spirochetes based largely upon morphologic differences (p. 151). The following classification, adopted by Bergey, is based upon that of Noguchi, but uses the name *Borrelia* for the genus which he originally termed *Spironema.*

Family I. Spirochaetaceae

Genus I. *Spirochaeta* (Ehrenberg, 1838). Large free-living fresh-water and marine forms. Type *S. plicatilis* (500 × 0.75μ), cylindrical, with regular spirals 1.5μ apart. Has an elastic flexible axial filament but no crista or flagella. Not dissolved by bile salts or saponin in 10 per cent solution.

Genus II. *Saprospira* (Gross, 1911). Large free-living marine and fresh-water forms; type *S. grandis* (100 × 0.8μ). Is divided internally into chambers by many transverse septa. Organism disposed in numerous relatively rigid undulating curves. There are no flagella nor is there an undulating membrane (crista).

Genus III. *Cristispira* (Gross, 1910). Large spirochetes parasitic in alimentary tract of oysters and other shellfish; type *C. balbianii* (Certes, 1882) (45 to 90 × 1.8μ with obtuse ends, cylindrical and composed of two to five large irregular flexures). Has a distinct and flexible longitudinal crest and an internal chambered structure like *Saprospira.*

Family II. Treponemataceae

Genus I. *Borrelia* (Swellengrebel, 1907). Type *B. gallinarum* (*B. anserina* = syn.) (Sakharoff). Various sizes, no axial filament, no crista, or undulating membrane. All disintegrated by 10 per cent saponin and bile salts; 5 to 14μ in length × 0.5μ in width, flexible and snakelike. In man *B. recurrentis, B. novyi, B. duttonii, B. berbera, B. carteri, B. vincentii, B. buccale, B. eurygyrata, B. bronchialis, B. theileri* and many others.

Genus II. *Treponema* (Schaudinn, 1905). Type, *T. pallidum* (Schaudinn and Hoffmann). Shaped like a corkscrew, pointed ends, 8 to 14 × 0.3μ with from 6 to 12 turns of the spiral. With dark ground appears as silvery, delicate corkscrew in motion. Imperfect illumination may show the organisms as dots. Curves rigid while in *Borrelia* they tend to straighten out. Members of both these genera (German theory) composed of ecto- and endoplasm, the former being continued beyond the latter forming the attenuated ends.

Genus III. *Leptospira* (Noguchi, 1917). Type *L. icterohaemorrhagiae* (Inada and Ido). Seven to 14μ in length, 0.3μ in width, with pointed ends and a spiral amplitude of 0.45μ; one or more gently undulating curves. Terminal filament, axial filament, and undulating membrane absent. Resists 10 per cent saponin but is dissolved by bile salts. There are many pathogenic species. Progress is by rotary motion.

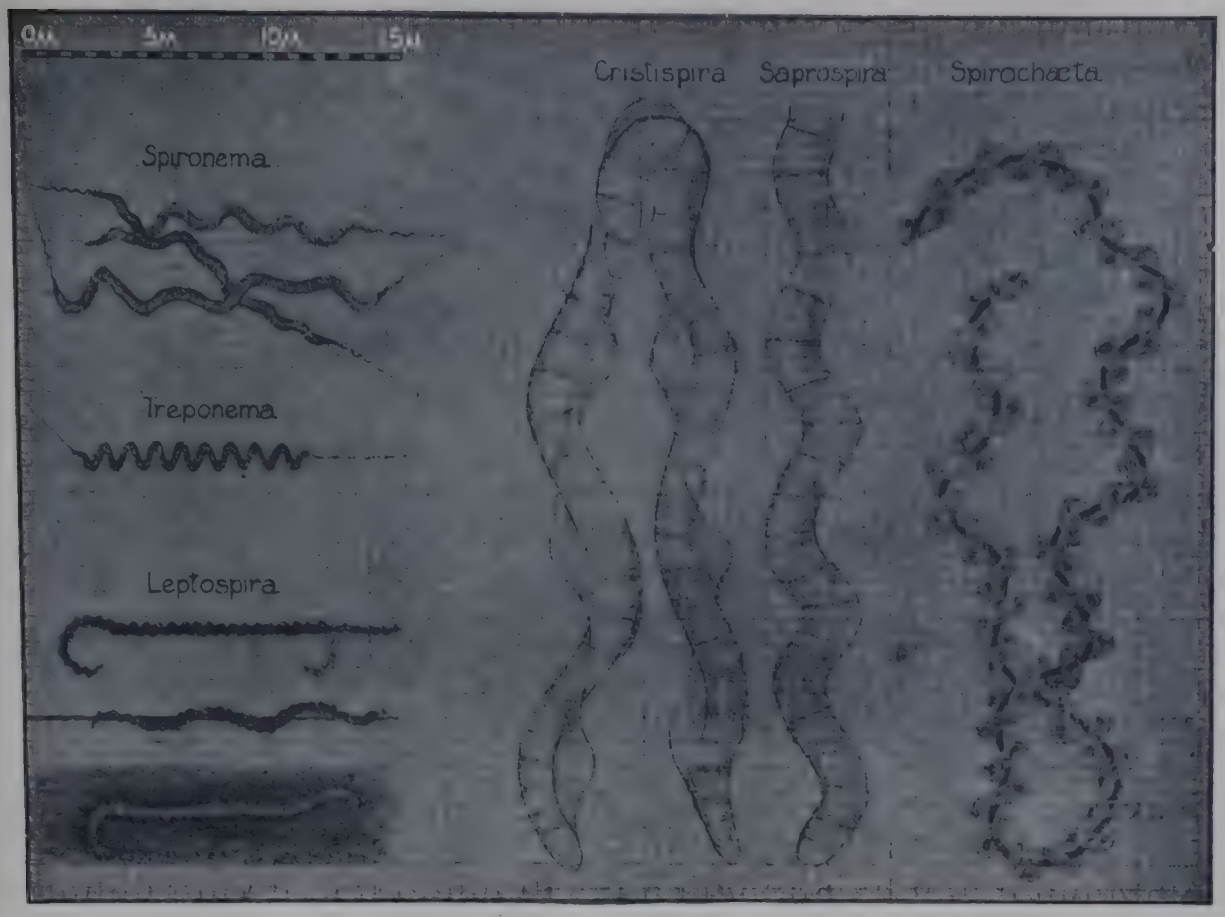


Diagram contrasting the characteristic features and relative proportions of *Borrelia* (*Spironema*), *Treponema, Cristispira, Saprospira, Spirochaeta,* and *Leptospira.* The scale in micra is given in the upper left-hand corner of the figure. (After Noguchi in *J. Exper. Med.*)

The Spirochaetaceae do not occur in man and are of no medical significance. The members of the genus *Borrelia* are often referred to as the blood spirochetes in contradistinction to the *Treponema* or tissue spirochetes. Intermediate between these two genera is the *Leptospira* group, the members of which have characteristics in common with both. The *Borrelia vincentii,* however, has not been demonstrated in the blood.

Formerly one of the two organisms which may cause rat-bite fever was included here in the genus *Spirochaeta* (*Spirochaeta morsus-muris*). This organism differs morphologically from other members of the group in the rigidity of its spirals. Unlike other spirochetes it possesses one or more flagella, and its motility resembles that of the vibrios. On this account Bergey considers that it should be placed in the genus *Spirillum* (*Spirillum minus*). Rat-bite fever, however, whether due to a streptobacillus or a spirillum, resembles other spirochetal infections clinically.

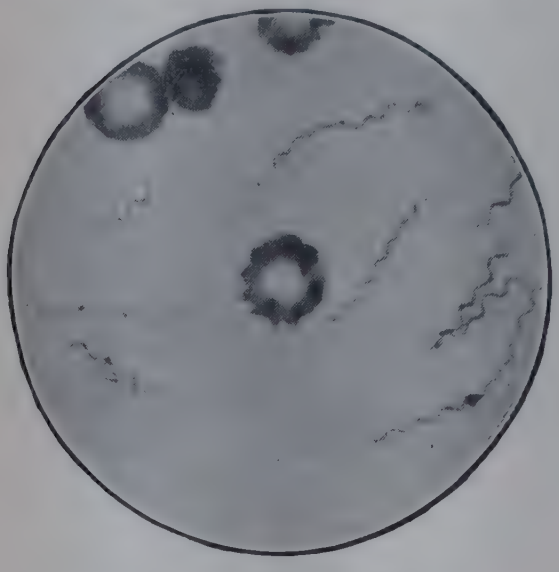
Some investigators have described granular forms which they believe represent a stage in the life cycle of the spirochetes. A filtrate containing such forms has been shown to be infective, but inasmuch as many of the flexible spiral forms may also work their way through bacterial filters, the assumption that the granules are a phase in the life cycle is not thereby proved.

Multiplication of the spirochetes is now known to be by transverse fission, as in the case of ordinary bacteria, and not by longitudinal fission as was formerly believed.

Spirochetes Causing Relapsing Fevers

Relapsing fever is an arthropod-borne, spirochetal infection which is widely distributed throughout Africa, India, and eastern European countries. Widespread epidemics have occurred in many other countries including the United States. The disease is characterized by a short febrile period (4 to 10 days) which begins and

ends abruptly and is usually followed after a week or two by a similar but milder paroxysm. In the African "tick fever" there may be as many as 10 such relapses, whereas in the European type of the disease there are rarely more than two or three. With each relapse there is a fresh invasion of spirochetes into the blood stream, where they persist until shortly before the crisis. During the remissions the organisms practically disappear from the peripheral blood, but they may be found in large numbers in the hyperplastic spleen in which they are apparently harbored.



Borrelia recurrentis from blood of a man with relapsing fever. (Kolle and Wassermann.)

There is no significant clinical difference in the disease as it occurs in the various countries. In some regions, however, it is spread by infected lice, and in others by infected ticks. Both louse-borne and tick-borne cases have been reported from the same region in South America. It has been shown that the spirochetes responsible can live in the bodies of bedbugs, and it is quite possible that these insects may also act as vectors.

The spirochetes causing relapsing fever have been differentiated into several more or less distinct types or species, of which the more clear-cut are *B. recurrentis* causing the (louse-borne) European disease, and *B. duttonii* causing African tick fever. Morphologically similar organisms are responsible for various arthropod-borne blood infections in fowls, cattle, and other animals.

Borrelia Recurrentis (*Spirillum obermeieri*) (*Obermeier, 1873*). This is the cause of the relapsing fever occurring in eastern Europe, and some investigators believe that it is identical with the spirochete responsible for the disease in India, and in Egypt and the northern part of Africa.

Transmission. The European infections are transmitted from one individual to another by lice. After an infected person is bitten by a louse, the spirochetes, which are taken into the alimentary tract of the louse, disappear within a few hours. The insect is apparently harmless for the succeeding four or five days. At the end of this time spirochetes reappear in the celomic fluid of the louse, which then remains infectious for two or three weeks. It is probable that organisms may be present also in the excreta through which the bite wound may be contaminated. Nicolle and others were able to produce relapsing fever in animals by rubbing their abraded skin with an emulsion of crushed, infected lice after having shown that infection was not produced by the bites of the lice or by the injection of their feces. It is generally accepted, however, that the disease in man is caused by contamination of a bite wound or a scratch by the material from a crushed louse or by its feces. It is believed that a reservoir of infection exists in certain small rodents.

Morphology. The organisms are variable in size, but average from 10 to 20μ $\times$ 0.4μ. They are flexible, and have from 4 to 10 open, irregular coils. They have an active corkscrew motility in fresh-blood preparations. They are easily stained by the usual bacterial

stains and by the Romanowsky blood stains, as well as by silver-impregnation methods. Individual organisms may have a beaded appearance, although a majority of them stain uniformly.

Cultivation. Noguchi succeeded in obtaining cultures of *Borrelia recurrentis* by using a medium containing agar, ascitic fluid, or blood, and a bit of fresh, sterile tissue, and incubating the inoculated medium anaerobically. Cultures have since been obtained in media enriched with serum or blood without the addition of fresh tissue. *B. duttonii* has been cultivated in the chick embryo.

Animal Inoculation. Monkeys, mice, and rats can be infected by subcutaneous inoculation. The disease produced resembles the human infection, and the spirochetes are demonstrable in large numbers in the blood during the febrile period. Guinea pigs are resistant to infection.

Immunity. With recovery from the disease due to *Borrelia recurrentis* a transient immunity develops, and the patient's serum acquires the ability to protect animals from subsequent inoculation. Bactericidal and agglutinating antibodies can be demonstrated. Relapses are frequent but are ordinarily less severe than the original attack, and may be explained by an inadequate formation of antibodies. The more resistant spirochetes survive and multiply, and again invade the blood stream. With each relapse the immunity is "stepped up" until recovery is complete. Phase variation of *Borrelia* may explain the relapses and eventual recovery from the disease. These spirochetes have been found in the blood of symptomless individuals, and such persons may be markedly responsible for the dissemination of the disease.

Laboratory Diagnosis of Relapsing Fever. During the febrile phase of the disease the spirochetes may be demonstrated in films of the blood stained by one of the ordinary Romanowsky stains, or by dilute carbolfuchsin. They may be seen in fresh preparations examined by darkfield illumination. They are sometimes found in the blood during the afebrile period. If they are not numerous, thick films should be prepared. If they are not found in smears, a mouse should be inoculated with the blood. Within 24 or 48 hours the spirochetes can be found in the blood of the mouse, if they were present in the inoculum. For demonstrating them in tissue sections, silver-impregnation methods are used. There is usually a well-marked polymorphonuclear leukocytosis in acute cases. The use of darkfield microscopy for diagnosis of spirochetal infections cannot be overemphasized. Staining technics are likely to alter the organism in such a way as to cause confusion.

Borrelia Duttonii. This spirochete is the etiologic agent of the relapsing fever prevalent in the western, eastern, and central regions of Africa (African tick fever). It is practically indistinguishable from the *Borrelia recurrentis* morphologically, but it is believed to be a different species. It is transmitted from one individual to another by a tick (*Ornithodorus moubata*) and possibly, in some cases, by other arthropods.

Spirochetes are found in the coxal fluid and feces of the tick, and human infection is caused by contamination of the tick bite by these excreta. The organisms have also been found within the eggs of infected ticks, in the nymphs, and even in the third generation. Leishman describes a breaking up of the spirochetes in the alimentary tract of the tick into small granules which penetrate the Malpighian tubules and the ovary. He regarded these granules as the infecting agents, and suggested that they represent a phase in the life cycle of the spirochete. However, according to Wenyon, this hypothesis has not been definitely substantiated.

African tick fever is similar to the louse-borne European relapsing fever in its clinical manifestations, but multiple relapses are more frequent. The diagnosis is established in the same way. *Borrelia duttonii* is more virulent for experimental animals than is *Borrelia recurrentis*.

Borrelia Novyi. This organism, found in relapsing fever cases in North America, has been differentiated from other responsible organisms by immunologic reactions. It is apparently transmitted by lice.



Borrelia novyi. (Todd.)

Other Spirochetes Responsible for Relapsing Fever. Other varieties of spirochetes have been described—*B. carteri* in the Indian relapsing fever; *B. berbera* in Egyptian and North African cases; *B. persica* in Persia and *B. babylonensis* (Brumpt) in Mesopotamia. These are not sharply differentiated from other types, however, and it is questionable whether they actually represent separate species.

A relapsing fever has been reported from Texas transmitted by *Ornithodorus turicata*. This tick inhabits certain caves which harbor various animals. Francis (1938) showed that these ticks survived and transmitted the infection after starvation for five years. A similar infection in California is transmitted by *O. hermsi*, and in British Columbia a *Dermacentor* is supposed to be concerned. Cases are, however, widely distributed throughout the west and with a variety of vectors. Brumpt notes the high degree of hereditary transmission in these ticks, and again suggests that these blood spirochetes may have been commensals for invertebrates in primitive periods, later infecting vertebrates.

Spirochetes Associated with Fusiform Bacilli

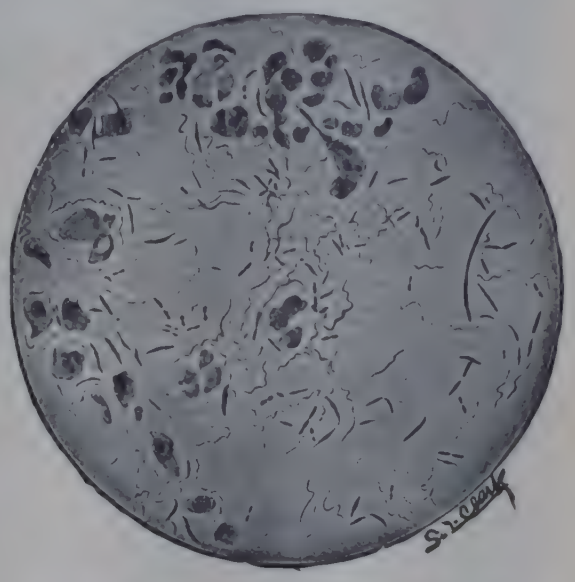
Borrelia Vincentii and Fusobacterium Plauti-vincenti. These organisms are found in small numbers in the mouths of most healthy adults, particularly around the gingival margins and in the tonsillar crypts. They are very numerous in the pseudomembranous ulcerative inflammations known as Vincent's angina and Vincent's stomatitis, and may complicate other types of ulceration, such as diphtheria, syphilis, or carcinoma.

They are practically always found together, but it is not yet definitely known whether their association is a symbiosis, or whether they represent different forms of the same organisms. Some investigators (Tunnicliff, Smith (1932), and others) have observed an apparent transformation from one type into another in cultures, while others have failed to do so and believe that these two organisms represent distinct species. It is convenient, however, since they are so closely associated in the lesions, to consider them together. Both are obligate anaerobes, and can be grown on enriched artificial media. Cultures have a fetid odor.

Borrelia vincentii is a slender, delicate spirochete with a variable number of shallow and regular undulations. In darkfield preparations from a lesion it is actively motile. It

stains readily with any of the silver-impregnation methods (Fontana's stain), and with Giemsa's stain, but may also be demonstrated by slightly overstaining with dilute carbol-fuchsin (1 to 5) or Loeffler's methylene blue.

Fusobacterium plauti-vincenti is a coarse, plump fusiform rod. It is usually from 5 to 7μ in length, but individual organisms may vary beyond these limits in the same preparation. They may be straight or slightly curved, and the ends taper to a sharp or dull point. In stained preparations the bacilli are characteristically beaded or banded. They can be demonstrated with the ordinary bacterial stains, and are generally Gram-negative. They have been described as nonmotile, but in some types a definite and even active motility has been observed.



Oral spirochetes. A smear from a case of Vincent's angina. (Coplin.)

Classification of Spirochetes and Fusiform Bacilli Found in Mouth. Attempts have been made by various investigators to differentiate the spirochetes found in the mouth, but their relationships are still confused. Noguchi described and cultivated types which he designated as *Treponema microdentium, T. mucosum,* and *T. macrodentium,* but there is some doubt as to the existence of the last two as independent species. Only three types of oral spirochetes can be recognized with accuracy on a morphologic basis; namely, the small oral *Treponemas, Borrelia vincentii,* and *Borrelia buccalis.* These oral spirochetes, when observed in the living state by darkfield illumination, present sufficient differences to be distinguished and identified (Hampp, 1945). Recent attempts to classify oral spirochetes by serologic methods show promise of obtaining a classification on firmer grounds (Hampp, 1946; Wichelhausen, 1946).

Considerable study has been given to the fusiform bacilli in recent years. On the basis of agglutination tests with 18 pure strains, Varney (1927) described 4 types and 2 subtypes. Smith (1932) described 3 types on morphologic appearance. Stanetz and Rettger (1933) divided 53 pure strains into 4 types on morphologic, cultural, and fermentative grounds. Hine and Berry (1937) on a similar basis, separated 104 pure strains into 3 types: *Fusobacterium nucleatum, F. polymorphum,* and *F. dentium.* The relation of the types in these classifications to each other is shown below.

	F. nucleatum	*F. polymorphum*	*F. dentium*
Varney	III	I and II	IV
Smith	III	II	I
Stanetz and Rettger	I	II	III and IV

Other discussion of the genus *Fusobacterium* is found in Chapter 5, p. 146.

Fusospirochetosis. Since the original description by Vincent of spirochetes and fusiform bacilli in cases of hospital gangrene, they have been found in a variety of pathologic conditions associated with putrid inflammatory ulceration. The commonest sites are around the gums, tonsils, and mucous membrane of the mouth. In these areas, lesions caused by these organisms may be confused with diphtheritic, syphilitic, or carcinomatous ulcerations, or spirochetes and fusiform bacilli may be present as secondary invaders in these ulcerous conditions. Infection may extend from the mouth and throat into the surrounding tissues, causing extensive necrosis, or into the middle ear, larynx, trachea, and bronchi. Less frequently any of the other mucous membranes may be involved—esophagus, colon, appendix, and rectum. The external genitals may be the site of fuso-

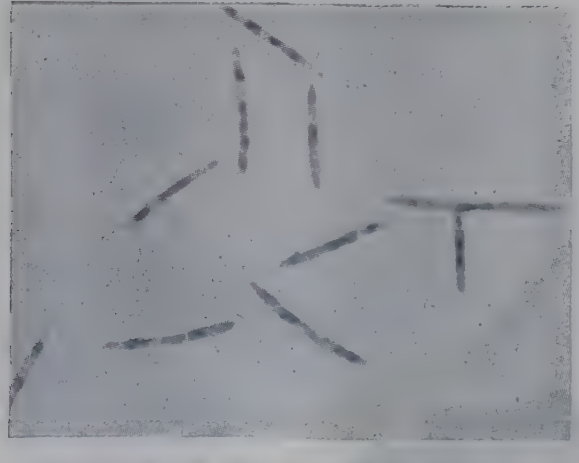


Borrelia vincentii. (× 1400.) Darkfield and stained smear of a pure culture. (Hampp.)

spirochetal ulcerations, either as a primary infection, or secondary to syphilitic or chancroidal lesions. Deep phagedenic ulcers may result. Postoperative and other skin wounds may become infected. Many cases of tropical ulcer fall into this category. Finally the bronchi and lungs may be affected, with the development of a putrid bronchitis or bronchopneumonia (particularly postoperative) which is often followed by abscess and gangrene. In these cases the organisms can be found in the sputum and their early recognition is imperative since without appropriate therapy the mortality is high—40 per cent according to Kline and Berger. These organisms are frequently found in pyorrheal pockets, and although they have no direct causal relationship to the condition, they undoubtedly aggravate it. Such areas when neglected have been aptly termed "anaerobic incubators." Spirochetes and fusiform bacilli practically always disappear from the mouth after extraction of all the teeth.

The ulcers on section consist of three distinct layers. The superficial layer contains necrotic cellular material and masses of mouth bacteria with varying numbers of spirochetes and fusiform bacilli. Below this is a layer of acutely inflamed tissue in which the fusiform bacilli predominate. The deep layer contains masses of spirochetes which appear to be the actively invasive agents. Streptococci usually are demonstrable throughout the lesion. There is little or no leukocytic infiltration.

Pathogenicity of Organisms Responsible. The role which spirochetes and fusiform bacilli play in the production of Vincent's infection is not entirely clear. Practically always other bacteria are found associated with them, and whether they are the primary etiologic agent or whether they are present as secondary invaders is disputed. Kritchevsky and Séguin believe them to be primary, and have produced local abscesses in animals and occasionally a generalized spirochetosis by the injection of pure cultures of spirochetes and fusiform bacilli. When either alone was injected, no lesions were produced. The invasion of pyogenic cocci was believed to be a secondary phenomenon. Smith (1932), however, believes that other anaerobic organisms, especially streptococci and vibrios, are also concerned in the infection. He was unable to produce lesions with cultures of any of the organisms separately, but only with mixtures of all of them. He has also shown the identity of the organisms from oral and



Fusobacterium plauti-vincenti. Pure culture in Douglas' broth, 3 days old. Stained with gentian violet. (× 1000.) (From *J. Bact.*, 34: 532.)



Electron microscope photograph of a small mouth *Treponema*. (× 16,000.) (Hampp and Wyckoff.)

from pulmonary lesions. By intratracheal injection into rabbits he produced typical lung abscess or gangrene both with sputum from human cases and with membrane from cases of Vincent's angina.

Laboratory Diagnosis. This depends upon the demonstration of the spirochetes and fusiform bacilli in stained smears or by darkfield illumination in perfectly fresh material. If possible the membrane should be removed and preparations made from the depths of the ulcer. The fact that these microörganisms are present in small numbers in the mouths of many healthy individuals must be remembered in interpreting the findings, and other pathologic conditions to which they may be secondary should be excluded. Sputum must be examined when fresh, since the spirochetes may be autolyzed within an hour or two. The peculiar sickening, slightly sweetish odor to the breath in infections of the mouth and lungs may suggest the etiologic factors. In fusospirochetosis there is no increase in the granular leukocytes in the blood, and there is occasionally a marked lymphocytosis.

Leptospira

Leptospira Icterohaemorrhagiae. This organism is the type species of the genus *Leptospira,* first described by Noguchi. These organisms are not so dis-

tinctly blood parasites as are those of the relapsing fevers, but are intermediate between them and the true tissue spirochetes. *L. icterohaemorrhagiae* is the cause of infectious jaundice (Weil's disease). The organism was first described by a group of investigators in Japan, where the disease is prevalent and severe. During World War I and World War II the organism was found in epidemics of the disease among the soldiers. These European strains were immunologically identical with those from Japan.

In some cases of a disease of dogs known as "canicola fever" an organism has been found which resembles *Leptospira icterohaemorrhagiae,* but which may be differentiated from it by various methods.

Symptoms of Infection (*Infectious Jaundice*) (*Weil's Disease*). The clinical picture of Weil's disease is not unlike that of yellow fever, in spite of the difference in the etiologic agent and in the transmission of the two diseases. The onset of symptoms is abrupt after an incubation period of 7 to 13 days, with rigors, fever, headache, vomiting, and muscle pains. The conjunctivae are hyperemic. Albuminuria is present. After from three to six days jaundice appears in 50 to 60 per cent of the cases, with enlargement of the liver and spleen, and a tendency to hemorrhages into the skin or mucous membranes. There may be symptoms of meningeal irritation or encephalitis. In the pre-icteric period the *Leptospira* are practically always present in the blood, but they disappear rapidly in the icteric stage. At this time antibodies make their appearance in the blood. The organisms are very numerous in the liver and in the kidneys; and in the latter organ they may persist for weeks after convalescence, during which time they are excreted in the urine. James H. Hutchison and colleagues (1946) reported the study of 17 proved cases (six fatal). The infection was among soldiers bathing in the River Arno during the summer of 1944. In all cases the onset was sudden with prostration, severe headache, and general muscular aching and stiffness. Icterus developed between the eighth and thirteenth days. Petechiae of the skin and mucous membranes were invariably found. Oliguria appeared at about the time of the onset of jaundice. Casts and red blood cells were common in the urine. In the fatal cases, postmortem examination showed marked renal changes similar to those following a hemolytic transfusion reaction. There was no necrosis nor degeneration of the liver cells. Canicola fever resembles Weil's disease clinically except that there is little or no jaundice.

The average mortality in clinically outspoken cases of Weil's disease is said to be about 20 per cent. However, studies of British workmen in occupations which involve frequent exposure to infection (sewer workers, miners, butchers) have shown positive serologic reactions in 25 per cent of such people and indicate that mild or subclinical infections must be relatively common.

Transmission. The infection occurs naturally in a high percentage of rats in various parts of the world, even in regions in which human infection is rare. The rat apparently remains healthy, but the organisms are demonstrable in the kidneys, and are excreted in the urine. Field mice have been found to be infected, and the organisms can be kept for long periods in living mice, but the natural reservoir of the organisms is the rat. Dogs are naturally infected with *L. icterohaemorrhagiae* as well as with *L. canicola.* Man is secondarily infected by contact with soil or food which is contaminated by the urine of affected rats. It has been demonstrated that the *Leptospira* can penetrate the skin even when there are no apparent abrasions, and it is believed by many that this is the usual atrium of infection. It is a recognized fact that the disease is particularly prevalent in wet

areas, and it has been suggested that a water-sodden skin facilitates penetration by the *Leptospira*. They have been shown to survive in moist soil or stagnant water for as long as three months. Infection may also occur through the digestive tract, however, and Inada believes that this is the usual way.

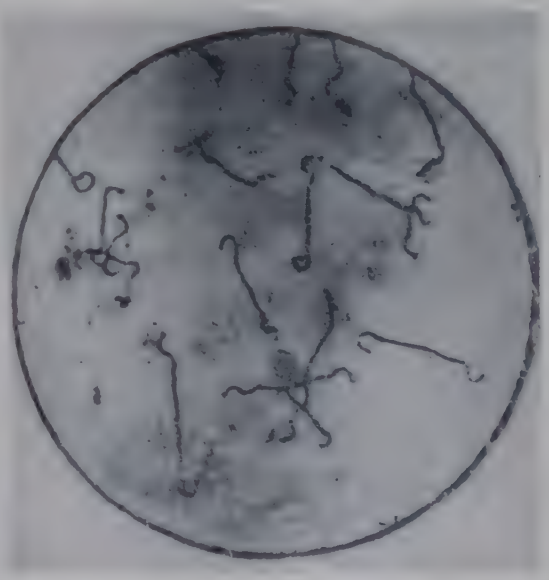
Morphology. The *Leptospira icterohaemorrhagiae* vary considerably in size—6 to 14μ (even 4 to 20μ) $\times$ 0.25μ. Noguchi showed that the threadlike body of the organism is made up of a large number of minute, tightly coiled elementary spirals. There are often several coarse, irregular undulations of the body, and one or both of the ends frequently show a characteristic hook. There are no terminal flagella. Propulsion seems to occur by a rotary motion on the long axis and by undulations. When only one end is hooked, progression is in the direction of the straight end. It is best seen by darkfield illumination, but can be demonstrated with Giemsa's stain or by one of the silver-impregnation methods.

Cultivation. These organisms can be cultivated on a variety of media whose essentials are a slightly alkaline reaction and a supply of native protein.

The *Leptospira* is able to pass through the ordinary types of Berkefeld filters (N, V, W). Inada has obtained filtrates in which no spirochetal forms were demonstrable, but which were infective for guinea pigs. He believes, therefore, that there may be a viable, granular form.

Animal Inoculation. Young guinea pigs are particularly susceptible to Weil's disease, and die within 10 or 12 days with characteristic pathologic lesions. The tissues are bile-stained, and the pleural and peritoneal surfaces are dotted with hemorrhages. The liver is enlarged and is teeming with organisms which are easily visible in a darkfield preparation. The virulence of this *Leptospira* is easily lost.

Immunity from Infection. By the end of the first week of the disease antibodies become demonstrable, and with their development there is a decrease in the number of organisms in the body. Convalescent sera will protect guinea pigs from an otherwise fatal dose of the *Leptospira*. The immune serum contains lysins which cause a breaking up of the organisms in the animal body (Pfeiffer's phenomenon), and *in vitro*.



Leptospira icterohaemorrhagiae, prepared by silver impregnation. The tightly wound spirals may be seen on close inspection. (From Gay: "Agents of Disease and Host Resistance," Courtesy, Charles C Thomas, Springfield, Ill.)

Laboratory Diagnosis. The *L. icterohaemorrhagiae* are present in the blood only during the first few days of illness. The organisms may possibly be seen in fresh preparations by darkfield illumination but it is usually necessary to inoculate a guinea pig (3 to 5 ml. of blood intraperitoneally) in order to demonstrate them. After the animal dies they can always be found in large numbers in an emulsion of the liver.

After the first week of the disease they should be searched for in the urine.

The centrifuged sediment can be examined directly, but this is not recommended, and by guinea pig inoculation. Inada has found that in practically all cases of the disease organisms appear in the urine by the twentieth day. If cultures of the organism are available the presence of antibodies in the serum may be tested by agglutination or protection tests or by the Pfeiffer reaction.

The agglutination test of Schüffner and Mochtar is the simplest method of establishing a diagnosis. Agglutinins appear in about 8 to 10 days following the onset of illness and may develop titers of 1 : 300,000 or more. See p. 287.

The *cerebrospinal fluid* shows minor nonspecific abnormalities in a majority of the outspoken cases (86 per cent of 92 cases examined or collected by Cargill and Beeson, 1947), regardless of the presence of symptoms of meningeal irritation. These were a slight to moderate increase in cells and, less regularly, in protein, and xanthochromia if jaundice was present. The color appeared sooner and in much greater intensity than in patients with corresponding degrees of jaundice due to obstruction.

Skeletal muscles (biopsy) may show changes which are regarded as characteristic (Sheldon, 1945) but not proved to be specific. These consist of focal areas in individual muscle fibers in which there are vacuole formation, loss of striation, hyaline necrosis, and later repair with proliferation of sarcoblasts. There was rarely hemorrhage and relatively little inflammatory reaction or fibrosis.

Vaccination and Serum Treatment. In Japan prophylactic vaccination with killed organisms has been tried with apparently good results. Immune sera have been obtained from horses and used in treatment. The results are said to be favorable if the serum is administered early in the disease. After the jaundice has appeared, however, serum treatment seems to have little or no effect. Recently it has been shown that penicillin is suitable for treatment, but that its value is limited if treatment is not begun until jaundice is established.

Leptospira Hebdomadis. This organism is the cause of seven-day fever, a disease which occurs in parts of Japan and resembles a mild attack of Weil's disease. The organism cannot be distinguished morphologically from that of Weil's disease, but Ido, Ito, and Wani were able to differentiate it by cross-protection tests and by the Pfeiffer phenomenon. They found that the organism was carried by field mice and transmitted to man by contamination of the soil with the urine of the infected animals. It is only feebly pathogenic for animals.

Leptospira Autumnalis. Similar organisms have been found in cases of the disease known as "autumn fever" in Japan. Koshina, Shiozawa, and Kitayama have differentiated two species by their serologic reactions. One was found to be identical with *L. hebdomadis*. The other, *L. autumnalis,* was more virulent and resembled the *L. icterohaemorrhagiae,* but did not correspond to either serologically.

Spirillum Minus

Rat-bite Fever Due to Spirillum Minus. Rat-bite fever is a term used to describe a specific clinical disease caused by the bite of a rat or other infected animal. The disease is due to either *Spirillum minus* or *Streptobacillus moniliformis*. Although certain differences in the clinical aspects of the two forms of the disease have been reported, it is only by demonstration of the causative organism that diagnosis can be accurately made. The infection caused by *Streptobacillus moniliformis* is discussed in Chapter 5 (p. 147).

Futaki and his colleagues (1916) first demonstrated *Spirillum minus* as one of the causes of rat-bite fever.

A week or two after the bite of an infected rat, fever develops which is relapsing in type and may continue for long periods. The site of the bite becomes inflamed, and the regional lymph nodes enlarge. After a few days a maculopapular rash appears which tends to recur with succeeding paroxysms of fever. Organisms are present in the local lesion and the adjacent lymph nodes, and occasionally in the blood in the acute stage. The disease is prevalent in Japan, and occasional cases have been reported from many countries including the United States.

In a carefully studied case attributed to cat-bite, Swyer reports that there were several relapses, in the second of which mice were inoculated and the presence of *Sp. minus* was established. At this time the patient was given neoarsphenamine but this drug failed to prevent severe recurrences. Following penicillin therapy, complete recovery took place. The manifestations at the site of the cat-bite were insignificant and there was no lymphangitis. Swelling of axillary and epitrochlear gland ensued. Marked myositis occurred and did not disappear until the penicillin was given.

Transmission. In an infected rat the organisms are found in the connective tissue, particularly around the lips, tongue, and nose. They have not been found in the saliva, and the transfer of infection by the bite appears to depend upon the existence of some break in the mucous membrane around the mouth. Mooser, working with experimental animals, has noted the frequent occurrence of infections in the eye and was able to find organisms in the conjunctival secretion. He suggests that this may be the source of the infection transmitted by the bite. Richter (1945) recorded 65 rat-bite wounds treated at Johns Hopkins Hospital from 1939 to 1943. Of the persons bitten 10.7 per cent developed rat-bite fever. Swyer (1945) notes that in England cat-bite rather than rat-bite is the more common cause of infection.

Morphology. The organisms are extremely variable in size. Most of them range from 2 to 5μ $\times$ about 0.2μ, but much longer forms are occasionally seen. The coils vary in number depending upon the length of the organism, and are uniformly spaced so that their crests are approximately 1μ apart. The body is relatively rigid, and one or more flagella are present at each pole. The organisms can be stained readily with the usual aniline dyes, or by one of the Romanowsky blood stains. Silver-impregnation methods are used to demonstrate them in the tissues. The motility observed in darkfield preparations is unlike that of *Leptospira,* and resembles the rapid, darting movements of the vibrios. The organism remains rigid and is apparently propelled by the flagella.

Culture. Futaki believed that he obtained growth of the organism in special media, but others have not been successful.

Immunity. Ido, Wani, and Okuda demonstrated the presence of bacteriolytic antibodies in the serum of convalescents.

Laboratory Diagnosis. Diagnosis of rat-bite fever due to *Sp. minus* depends upon the demonstration of the organism at the site of the bite or in material aspirated from a regional lymph node. Occasionally the organisms may be found in the blood in early cases. Rarely they may be seen in smears from the material, but they are more readily demonstrated by animal inoculation. Guinea pigs are susceptible and usually succumb to the infection. The organisms can be demonstrated in the blood without difficulty. Rats and mice are also susceptible, but the blood invasion is transient, and they rarely, if ever, die from the infection.

Treponema*

Treponema Infections (*Treponemiases*). Treponema infections are sometimes referred to as treponematoses but the term "treponemiases" is preferable. Three treponema diseases, exclusively affecting man, are among the most important of human infections: syphilis, of world-wide distribution; yaws, almost entirely confined to the tropical belt; and pinta, a very important disease of Mexico, Central America, and northern South America. In Mexico 11 per cent of 2,500,000 persons examined had pinta (carate).

Historical Data. There seems to be satisfactory evidence that pinta existed among the Aztecs at the time of Cortez—letters having been written to the Spanish king referring to the prevalence of a disease considered as leprosy; the natives called those affected "carates." The need of hospitals for such patients was emphasized. The vitiliginous spots very occasionally reported as evidence of pinta in persons from the United States and from Africa should, probably, be attributed to yaws or syphilis.

As regards yaws there is every evidence that this was a common disease of African slaves. It was brought to Europe by the Portuguese 50 years before Columbus discovered the New World and to America by African slaves, imported into Hispaniola in 1502 to replace the fast-vanishing native Arawaks. In a book on North Carolina, written by Brickell in 1737, descriptions of yaws in slave women are given, and note is made of the transfer of this disease to white men, causing many of them to lose their noses. About the same time, Colonel William Byrd, in the Westover Manuscripts, reported a disease, which caused undermining of the foundations of the nose, as being common among white men on the southern side of the North Carolina-Virginia boundary line.

Our greatest American medical historian, Garrison, devotes almost four pages to the origin of syphilis, and accepts the views of Sudhoff that syphilis prevailed in Europe before 1492. Much emphasis has been placed on the findings of syphilitic bones in American Indian burial mounds. Ales Hrdlicka, the great anthropologist, in an article on the diseases of the Indians (1932) states: "Notwithstanding some claims to the contrary, there is as yet not a single instance of thoroughly authenticated pre-Columbian bone syphilis." Quite recently an eminent historian, Samual E. Morison, stated that he has thoroughly examined the records of the voyages of Columbus and has found no entry in the logs of the various ships of the first and second voyages relative to the existence of any such disease as syphilis occurring in the companions of Columbus. He stated that while this is only negative evidence, yet it is so emphatically negative as to require consideration. The proponents of the New World origin of the disease make much of the syphilis of the Pinzon brothers, particularly Vincenti Pinzon, pilot of the Nina, the ship on which Columbus returned to Palos. Notwithstanding evidence submitted by other authorities, Morison expresses the belief that syphilis was introduced into Europe by women aborigines brought to Spain from America on Columbus's second voyage.

*This section on "Treponema" (pp. 162–167) was written by Rear Admiral E. R. Stitt.

It would seem that we may consider pinta as originating in America, yaws in Africa or, possibly, in the East Indies and the Polynesian-Melanesian Islands, and syphilis as an Old-World disease steadily advancing and, as considered by Lambert, conquering the yaws spirochete when introduced and thoroughly established in an area.

Immunity to Syphilis. The experiences of American physicians in Guam, and of the British in Fiji, evince the fact that a previous attack of yaws, particularly in childhood, confers immunity to syphilis. A report by the medical officer at Guam, in 1939, states that no case of syphilis among native women prostitutes has been found, notwithstanding the abundance of gonorrheal infections in such women. Nichols and Daniels, from the Fiji Islands, state that syphilis is unknown among the Fijians, although ample opportunity existed to contract it from the Europeans and Hindus. Turner found that rabbits inoculated intratesticularly with *T. pallidum, T. pertenue,* and *T. cuniculi* (the cause of a venereal disease in rabbits) and, six months later, with heterologous strains, gave evidence of immunity. The homologous-strain experiments showed a higher immunity.

Pathology. Generally considered, in human beings all three treponemiases start as a papule. The papule of syphilis, by reason of the change produced in relation to the blood vessels of the corium, tends to bring about necrosis of overlying parts and, later, ulceration. The papule of yaws develops rapidly into a granulomatous lesion (frambesioma) through hyperplasia of the cells of the Malpighian layer, giving a lobulated, elevated growth. The papule of pinta tends to flatten out, giving a leaden-blue spot (pintid), which eventually loses color and, in the terminal stages, is white. The striking change in the pintid is due to the tremendous disturbance of the pigment-bearing cells which tend to leave the basal-cell layer, at the bottom of the epithelial layers, to invade the overlying epithelium and, also, to penetrate the superficial corium. There is a tendency for the pigment cells invading the corium to form a barricade-like layer. Atrophy and absence of pigment characterize the histologic structure of the white pintid.

The similar mutilating lesions attending the late stages of yaws and syphilis are regarded as of allergic nature. Chesney emphasizes the lymphocytic and monocytic cellular response to syphilitic infections, rather than the polymorphonuclear invasion of other infections. Stokes points out that vascular localization is almost a tropism of the syphilitic spirochete, and most histologists have reported perithelial cellular accumulations which obstruct the lumen of the blood vessels of the corium.

Selective Localization of Treponemas. In 1907, Schüffner reported that in Levaditi-stained sections of yaws' lesions the treponemas were to be found in the epithelium and not in the corium, where they are found in syphilitic lesions. Marshall, in the Philippines, shortly afterward confirmed this finding and emphasized the presence of the treponemas in the superficial part of the papillae as well as in the interpapillary down-growth of the Malpighian cells (interpapillary pegs). Goodpasture (1923) made a careful study of the location of the treponemas in yaws, and noted their earliest appearance in the superficial layers of the

papillae; he felt that it was from this source that the invasion of the epithelium occurred.

In pinta the localization of the treponemas is in the epithelium, and not in the tips of the papillae or other parts of the corium. Ash and Spitz (1945) state that *T. carateum* is most easily found in the stratum spinosum and stratum corneum, rather than in the rete mucosum, and the organisms are especially numerous in follicles plugged by keratin debris, suggesting the role of the follicles in infection.

Superinfection. The early European slave traders of West Africa noted the wide prevalence of yaws among the natives and, furthermore, described a practice among them of producing in children, by auto-inoculation, a full crop (secondaries) of yaws' lesions not showing a wide generalization, which they considered desirable for the children. In the Philippines, Schöbl showed that the generalization common in man was only rarely noted in monkeys primarily inoculated in the scrotal or eyebrow regions, but upon reinoculation generalized yaws ensued. To this he gave the name "superinfection." One of the most valuable articles on human experimentation with yaws is that of Charlouis (1897). With an inoculation needle he made a pocket in the epidermis—not drawing blood. Into this pocket he inserted material taken from yaws tubercles. At first he carried out auto-inoculations, and these were successful in the three men who had well-developed yaws. Using these subjects as donors he inoculated 32 Javanese prisoners with success in 28 cases. In the successful cases, papules developed in about two weeks followed by secondary yaws in about three months. These experiments were undertaken to prove the contagiousness of yaws. In one experiment Charlouis successfully inoculated a yaws patient with syphilis, but as auto-inoculation of yaws is usually successful this might have been expected. Sellards, Lacy, and Schöbl (1926) carried out superinfection inoculations undertaken from four to six weeks after the primary infection. There is nothing definite as to the length of the immunization period, but it is certain that it differs from that of syphilis. Reisser states that if syphilitic material is inoculated on successive days the take continues for 10 to 15 inoculations, but, subsequently, only abortive, atypical, or negative results are obtained. Leon-Blanco (1940) inoculated 28 volunteers, including himself, with pinta treponemas. Of the five who had pinta, in only one was the inoculation successful; the healthy individuals became infected. Three syphilitics developed an initial papule but the secondary eruption was scant and atypical.

Animal Experimentation. Turner's experiments as to the different response to yaws and syphilis treponemas emphasized marked induration and enlargement of the testicle of the rabbit inoculated intratesticularly with *T. pallidum* while that of the rabbit inoculated with *T. pertenue* remained soft and only slightly enlarged and showed miliary granules, particularly located on the surface of the tunica vaginalis. Louise Pearce (1938), in a concise presentation of results of experimental inoculations of various animals with syphilis, notes that the disease in the rabbit combines the elements of both acquired and congenital syphilis of man. The articles by Turner and Pearce in the 1938 A.A.A.S. symposium on syphilis deserve particular attention.

As regards pinta, the reports of animal inoculations are conflicting. One of the leading investigators, Leon-Blanco (1945), describes a successful scrotal inoculation of one out of four rabbits. The nature of the virus was proved by the development of pinta in a man inoculated with treponemas from the scrotal lesion of the infected animal. In a later article, Leon-Blanco states that successful inoculation in experimental animals can be proved only by the development of pintids in human beings inoculated with material from the lesions of the animals.

In 1903 Metchnikoff and Roux produced, in a chimpanzee, a chancre which was followed by regional enlargement of lymphatic glands and a subsequent generalized papular eruption. The development of the chancre, lymphadenitis, mucous patches, and papular eruption followed the typical course of syphilis in man. Schöbl, by inoculating cynomolgus monkeys by scarification of eyebrows or scrotum, obtained lesions similar to those of yaws in human beings. He also found that yaws-infected monkeys acquired a certain degree of immunity to syphilis.

Treponema Parasites. The description of *Treponema pallidum,* given in the text under syphilis, will answer for the treponemas of yaws and pinta.

Noguchi reported the cultivation of the parasite of syphilis on a special medium, incubated anaerobically, from which he produced luetin, considered as analogous to tuberculin. This claim has not been substantiated in recent research. The problem of cultivation is still being actively investigated. Filtrates from material containing syphilitic treponemas have proved to be negative for *T. pallidum* by inoculation experiments. However, the work of investigators (lucidly presented by Pearce (1938)) in the unapparent syphilis of the mouse suggests a prespirochetal phase attributable to inoculated filtrates. Zinsser suggests that culture strains belong either to a different species of treponema or are dissociated forms of *T. pallidum*. The great syphilologist, Sir Jonathan Hutchinson, who emphasized the triad of interstitial keratitis, Hutchinson's teeth, and a characteristic physiognomy for congenital syphilis, held to the end of his life the view of unity for syphilis and yaws. When a very old man he personally investigated cases diagnosed as yaws in South Africa and, later, in Ceylon, and stated that he had seen cases of syphilis in England and France which resembled those labeled yaws. Garrison states that Sir Jonathan had observed more than a million cases of syphilis. In one of his articles he gave us the aphorism that Englishmen who contracted yaws in the colonies came home with syphilis. Admiral Butler, in his book on syphilis, was the pioneer advocate of this view in the United States. Captain Holcomb in numerous articles on syphilis, yaws, and pinta is our best authority on history. His article on the antiquity of congenital syphilis (1941) shows that Bernard de Gordon (about 1308) described congenital syphilis as a form of leprosy.

Syphiloids

From the seventeenth to the middle of the nineteenth century three syphiloids were described: sibbens, radesyge, and button scurvy. In this group also would, apparently, belong a treponema disease carefully studied by Hudson and called by the desert Arabs, bejel.

Sibbens. The syphilis-like disease first appearing during Cromwell's invasion of Scotland was given the name "sibbens" (Scotch for "wild raspberry"). Its prevalence was greatest around 1725, but it continued in the Highlands until 1840. It resembled a severe form of syphilis tending toward ulceration of the throat and palate. It was supposed to be transmitted through the common use of pipes and spoons as well as through sexual intercourse and skin contact. Physicians who had practiced in the Tropics maintained that the disease resembled yaws.

Radesyge. This disease was reported as occurring first in Norway and Sweden in about 1720. The descriptions of the clinical manifestations would indicate that there was a confusion of several diseases—yaws, syphilis, lupus, scabies, and leprosy. Both sibbens and radesyge were spread not only by sexual intercourse but by skin contact and by eating and drinking from common receptacles. The early lesions, like those of bejel, appeared in the mouth and throat. Some observers thought radesyge resembled yaws, except that the former tended to terminate fatally while yaws usually terminated favorably.

Button Scurvy. Hirsch considered button scurvy as more nearly resembling yaws than did sibbens or radesyge. It prevailed in Ireland from 1823 to 1851. It was very contagious and was especially prevalent in those who washed linen soiled by discharges from the sores. It was described as breaking out in nodules which grew into tumors varying from the size of a pea to that of a nut. The covering of the growth tended to be encrusted. There were lesions of the palms and of the inner surfaces of the arms and legs. There were also condylomas of the genital region. Like yaws, button scurvy lasted many months but usually terminated with little effect on the general health.

Bejel. This disease, chiefly affecting the children of the desert Arabs in Syria, has been splendidly described by Hudson. A similar disease, reported by MacQueen (1934) as syphilis insontium, occurs in the desert Arabs of Palestine. Syphilis among primitive races shows itself preponderantly as a disease of children, as does yaws. The most characteristic feature of bejel is the early appearance of grayish patches on the buccal mucous membrane, followed by lesions of the genitals and circinate papular eruptions of the trunk. Adults who contract the disease from children tend easily to develop the mutilating lesions of tertiary yaws or syphilis. Infection seems to be through the wooden drinking bowl used in common and from the prodigal practice of kissing. There is absence of circulatory and nervous symptoms and abortion due to bejel is rare.

Syphilis, Yaws, or Pinta?

Treponemas were first reported from a case of plantar hyperkeratosis in the clinic of Braulio Saenz, in Havana, Cuba, in 1938. This type of case had for years previously been diagnosed and treated as syphilitic hyperkeratosis. Moss and Bigelow (1922), in the adjoining island of Santo Domingo, had diagnosed similar cases as "Clavus," a common plantar hyperkeratosis following yaws.

Donald H. Baker, a medical missionary in the Belgian Congo, has reported (1942) syphilis to affect the Negro tribe in the region of the hospital, only 1 per

cent of those under 30 years of age showing yaws lesions. A tribe 20 miles distant gives a 50 per cent incidence, while the Topokes tribe, still more distant from the hospital, shows only a rare case of syphilis but much yaws. This situation presents an opportunity for a dermatologist, skilled in the pathologic technics of biopsies and sectioning, to study the conflict between *T. pallidum* and *T. pertenue*. Subsequently such an expert on yaws-syphilis should study the pinta of Mexico or Colombia.

Syphilis

Treponema Pallidum. Schaudinn and Hoffmann, in 1905, first described this organism which they demonstrated in the primary lesion and in the swollen lymph nodes in syphilis.

Morphology. *T. pallidum* is a slender, delicate spirochete ranging from 3μ to 15μ (average 10μ) in length by about 0.3μ in width, with tapering ends. There are from 6 to 12 relatively rigid, tightly coiled, small spirals regularly spaced about 1μ apart. Many individuals show a bend in their long axis. The organisms are best seen in darkfield preparations, in which they appear as silvery white coils which remain sharp and white when the focus is changed. While in motion the spirals appear continuous, but when at rest they often present the appearance of a series of silvery dots and dashes. Progression is due primarily to a rotary movement with slight undulations, with some expansion and contraction of the coils. Motility is less active than that of most of the spirochetes. This organism cannot be stained with the ordinary aniline dyes, but is well demonstrated by prolonged immersion in Giemsa's stain, or better by Fontana's silver impregnation method. Levaditi's method is recommended for sections.

The organism is not filtrable. It has been claimed on morphologic grounds that a granular or filamentous "prespirochete" phase occurs, but evidence of this has not been substantiated.

Pathogenicity for Animals. Monkeys are susceptible to syphilis, and typical primary and secondary lesions may develop. These animals may be inoculated subcutaneously, or by rubbing material on a scarified area on the eyebrows or genitals. In rabbits a syphilitic orchitis may be produced by intratesticular injection of syphilitic tissue or scrapings from a chancre. Occasionally a chancre-like skin lesion appears in from three to six weeks at the site of the puncture. Inoculation into the anterior chamber of the eye



(*Left*) *Treponema pallidum*. Stained smears. (After Noguchi in *J. Exper. Med.*)
(*Center*) *Treponema pallidum*. Darkfield. (After Noguchi in *J. Exper. Med.*)
(*Right*) *Treponema pallidum*. Fontana's stain. (After Noguchi in *J. Exper. Med.*)

causes a keratitis and iritis, and localized lesions elsewhere can be produced occasionally. Living spirochetes can be found in large numbers in the lymph nodes over a long period of time. In working with rabbits, it is important to recognize that they are subject to a natural venereal disease, the etiologic agent of which (*T. cuniculi*) may be morphologically indistinguishable from the *T. pallidum*.

Clinical Manifestations. Clinically the disease is divided into three more or less clear-cut stages, the manifestations of which depend in part upon the distribution of the organism, and the reaction of the tissues to its presence. One or more of these phases may be absent, and in some cases there may be no manifestations of the disease for many years. In the so-called "latent" cases the diagnosis depends solely upon serologic reactions.

After an incubation period of from three to six weeks a *primary sore* or chancre appears at the site of inoculation, and the regional lymph nodes enlarge. Spirochetes are present in these areas in large numbers, and from here they become widely distributed throughout the body. They occur in the blood stream, although not in sufficient numbers to be demonstrated directly. It is believed that the organisms gain entrance to the nervous system at this time in those cases which later develop neurologic manifestations.

Characteristic lesions on the skin and mucous membranes mark the onset of the *secondary stage* of the disease. Spirochetes are present, and can be demonstrated, in these areas. By this time characteristic serologic changes have developed in a great majority (95 per cent) of the cases, and are relied upon to confirm the clinical diagnosis.

After a variable length of time the secondary manifestations disappear. The spirochetes become much less numerous in the body, but generally persist in localized areas with or without the development of symptoms. Such a nidus may occur in almost any tissue in the body, and may produce gross pathologic lesions within a short time, or only after many years. As a result of these peculiarities in the infectious process, the manifestations of *tertiary syphilis* are of many different types, and occur almost anywhere in the body. The symptom complex of many unrelated diseases may be simulated by a syphilitic infection, and can be differentiated only by serologic tests. While spirochetes may be present in these tertiary lesions for many years they are usually sparse and are not easily demonstrated even in tissue sections. The organisms are frequently harbored in the lymph nodes which have been shown to be infective for rabbits, even in latent cases many years after the initial infection. Lansford and Day (1934) have tested the infectivity of the nodes in a series of Wassermann-positive cases in different stages of the disease. In the primary cases glandular transfer was positive in all; in all patients receiving treatment at the time infectivity was negative. In late cases, however, whether or not previous treatment had been given, transfer was successful in only about 35 per cent. These results indicate that failure to obtain infection in rabbits by inoculation from human lymph nodes is not a reliable criterion of cure, as has been suggested.

It has been claimed, chiefly upon clinical observations, that certain so-called *neurotropic strains* of *T. pallidum* possess a particular affinity for the central nervous system. There is, however, no conclusive proof of this contention, and the factors determining the type and location of the tertiary manifestations are not well understood.

Syphilis may be *congenital*, in which case the spirochetes may occur in great numbers in the internal organs, particularly the liver. They have been demonstrated in the blood, and are present in the placental lesions. Blood from the cord may show a positive Wassermann reaction. Adequate treatment of the mother before or during pregnancy will prevent the disease in the child, and if the fetus is already infected may combat the disease *in utero*.

Immunity. The problem of immunity in syphilis is but little understood. At no stage

of the disease are the ordinary antibodies such as agglutinins, or bacteriolysins, demonstrable in the serum according to most observers, and the serum does not possess any protective power for animals. In the tertiary stage allergic skin reactions may be obtained with spirochete extracts (luetin) and to other protein extracts, but they are not specific, and the luetin reaction is no longer utilized as a diagnostic procedure.

Serologic Reactions. Serologic changes develop, however, which are of the highest diagnostic importance, although their nature is not understood. Within from two to four weeks after the development of the primary lesion the serum acquires the property of reacting specifically with an emulsion of certain lipoidal substances from normal tissues. This reaction may be manifested by the occurrence of flocculation in a mixture of serum and lipoid emulsion, and by the demonstration of fixation when complement is added to such a mixture. This altered reactivity of the blood is present in practically all cases in the secondary stage of the disease and in untreated congenital cases, but may be absent in a minority of the tertiary and latent cases. When the central nervous system is involved the reaction is demonstrable in the spinal fluid although the blood reaction may be entirely negative.

Details of the Kahn flocculation test, the Kolmer complement-fixation test, and tests using cardiolipin antigen are given in Chapter 10.

The American Serological Conference has recently compared the results of the different tests on the basis of their sensitivity and specificity, and has concluded that flocculation and complement-fixation tests are equally reliable when properly performed. Although the test is practically specific, there are some 40 other conditions in which positive reactions may be obtained. In yaws the percentage of positive results seems to be as high or higher than in syphilis; and "false positives" are obtained frequently in leprosy and malaria.

This serologic change is associated in some way with the presence of spirochetes in the body and is not an index of immunity. The actual defense mechanism depends primarily upon some obscure alteration in the capacity of the tissue cells to react to the virus rather than upon humoral antibodies. The immunity which develops is rarely (if ever) effective in ridding the body of organisms but is usually sufficient to prevent superinfection. Although it seems to persist for some time after the disease is apparently cured, it is not life-long since a second attack of the disease may occur.

The problem is complicated by our inability to determine when the patient is actually cured. Various criteria of cure have been suggested, but none is entirely satisfactory. Syphilitic individuals are refractory to a superinfection, and the development of a reinfection has been regarded as a proof of cure. It is difficult, however, to prove that reinfection has not taken place, since many cases of infection occur without a recognized local lesion. Furthermore, it is possible that sufficient immunity to prevent reinfection may persist for some time after a patient is cured. The demonstration of lymph-node sterility by animal inoculation is so inconstant in human beings that it seems to be of little value as an indication of cure (Moore, 1935).

Laboratory Diagnosis. In the primary stage of the disease the diagnosis is established by demonstrating the *Treponema pallidum* in material from the chancre. To obtain material for examination the lesion is cleansed with alcohol and allowed to dry. The margin of the ulcer is rubbed with gauze, or scraped gently with a scalpel so that serum may be obtained from the corium. If bleeding oc-

curs the surface should be wiped gently until the exudate is clear. Gentle squeezing is permissible. Material may also be obtained by washing the lesion, and applying a Bier cup or a test tube which has been warmed so that upon cooling a partial vacuum develops.

This exudate should be examined fresh with darkfield illumination. The *T. pallidum* is easily seen, but must be distinguished from other nonpathogenic spirochetes (particularly *T. refringens*) by its tight corkscrew spirals and relatively sluggish motility.

When the material cannot be examined immediately, thin smears can be made and stained. Fontana's method is excellent. The Warthin-Starry method is good. The India-ink method of Burri is simple and can be recommended. The material is mixed with a small drop of drawing ink, and thin smears are made as for blood. When dry they are examined with the oil-immersion lens, and the spirochetes stand out as white spirals against a dark background.

As the *primary* lesion begins to heal the organisms become less numerous and may be difficult to demonstrate. At this stage, however, they are present in large numbers in the regional buboes, and can be found in the fluid obtained by gland puncture. Although spirochetes may occur in the blood in the primary stage of the disease, their demonstration here is not a practicable diagnostic procedure. The fact is of great importance, however, in selecting a donor for transfusion, since syphilis has been transmitted in this way in a number of instances. At this stage reactions to serologic tests are usually negative, and the condition may escape notice unless the donor is carefully questioned and examined.

In the *secondary* stage the spirochetes occur in the serous exudate obtained from mucous patches or from skin lesions. The spirochetes which occur in normal mouths (*T. microdentium*), however, cannot be distinguished morphologically from *T. pallidum,* but at this time the reactions to serologic tests are practically always positive, and the demonstration of the organisms is not necessary for diagnosis. In the *tertiary* lesions they are usually sparse, although they have been found in sections from a number of different tissues.

After the primary stage the complement-fixation and flocculation reactions in the serum are relied upon for diagnosis.

When *central nervous system* involvement is suspected the spinal fluid must be examined, even though the blood reaction may be negative. In addition to the serologic tests the following examinations are particularly important: (1) cell count; (2) globulin and total protein estimation; (3) quantitative estimation of glucose; (4) colloidal gold or mastic tests. These procedures are described in Chapter 35. No patient with syphilis should be considered cured until the spinal fluid as well as the blood has been examined and found to be normal.

Yaws (Frambesia)

Etiology. Yaws is an infectious, highly contagious, nonvenereal disease, limited to the Tropics and caused by an organism which is morphologically indistinguishable from, if not identical with, *T. pallidum* of syphilis. Blacklock refers to frambesia as "tropical syphilis," and lists the term *T. pertenue* as a synonym of *T.*

pallidum. The organisms are found in large numbers in the serous discharge from yaws' lesions and in the lymph nodes. They also occur in the blood, as shown by inoculation experiments.

Animal Inoculation. Monkeys and rabbits can be infected by inoculation with discharges from yaws' lesions. Schöbl (1928) obtained primary lesions in monkeys by inoculation of the eyebrows or (more readily) of the scrotum. By a reinoculation while the primary lesion was healing (superinfection) he obtained a generalized secondary eruption closely resembling yaws in man. In rabbits most observers have described dry lesions following skin inoculation with yaws, whereas in syphilis inoculations the lesions resemble chancres. Turner and others reported that the epididymo-orchitis in rabbits following inoculation with yaws is much less marked than that due to syphilis inoculation.

Epidemiology. Yaws is limited to the Tropics, and is especially prevalent in the West Indies, Africa, southeastern Asia, the East Indies, and the Pacific Islands. It is almost exclusively confined to members of the colored races. It is largely limited to rural districts in which syphilis is rare, whereas in the towns yaws is uncommon and syphilis (venereally acquired) is prevalent. Infection is usually acquired innocently in childhood. The spirochetes enter the body through some cut or abrasion of the skin, either by direct contact with discharges from the lesions or indirectly, particularly through the agency of flies. Kumm and Turner (1936) showed that rabbits could be infected with yaws by scarifying the skin and exposing them to *Hippelates pallipes* which had fed on infective discharges (see p. 702). These flies were observed in swarms feeding on the discharges from yaws' lesions. In Jamaica the curve of incidence of new cases parallels the rainfall and (consequently) the prevalence of *Hippelates*. The spirochetes apparently do not penetrate the unbroken skin. The rarity of the disease in white children may be explained in part by the fact that they are kept clothed and clean. The epidemiology of yaws and pinta is almost parallel, but it would seem that the contact must be more prolonged in pinta—more like that of leprosy.

Clinical Picture. A primary, secondary, and tertiary stage of yaws can be distinguished. The *primary lesion* (mother yaw) develops three and a half to four weeks after experimental infection (Sellards) and one to two months after natural infection. It appears as a small papule which attains a diameter of about 1 cm. or more and becomes covered with a crust of dried, serous exudate. If the crust is removed it reveals a fungoid, yellowish or reddish tubercle which exudes a serum containing spirochetes (frambesia). Irregular fever, malaise, joint pains, and nocturnal headaches precede the appearance of the primary lesion which is usually located on the legs. This may regress spontaneously after a few weeks or it may persist for many months. There is a regional, and later a general, glandular enlargement. From two to three weeks after the primary lesion appears, the Wassermann reaction becames positive.

Six to 12 weeks after the primary lesion a generalized *secondary eruption* develops, first as small, dry, scaly patches in which small papules soon appear. These enlarge and resemble the primary lesion. There is a recurrence of fever and constitutional disturbances. The lesions itch but are not sensitive or painful except on the palms and soles. The disease is called "crab yaws" when the lesions occur on the soles because of the resulting peculiar, awkward gait. There may be successive crops over a period of several months or years. The individual lesions usually regress after several weeks, leaving a thickened, subsequently pigmented scar.

In about 8 to 10 per cent of those afflicted the lesions persist as chronic ulcers (*tertiary stage*) which may invade the neighboring tissues extensively. Osteitis and periostitis are common and may involve the fingers as in syphilis, causing marked deformity. Other late lesions include: gangosa, juxta-articular nodules, and goundou.

The term *gangosa* is applied to a chronic ulceration resembling that sometimes seen in syphilis, which starts in the palate and gradually destroys the soft parts and bones of the roof of the mouth and the nose (but spares the upper lip), causing frightful mutilation.

Juxta-articular nodules of fibrous tissue, varying in size from that of a pea to a small orange, often occur on the extensor surface of the limbs, especially about the joints.

Goundou, probably also a tertiary manifestation of yaws, is characterized by a progressive, symmetrical enlargement of the nasal processes of the superior maxillae, which eventually form large projecting tumor masses.

Relation to Syphilis. The exact relationship of yaws to syphilis is still in controversy. Those who maintain that the diseases are distinct point out differences in clinical manifestations and in the lesions in experimental animals. They have emphasized: (1) The absence of mucous patches in yaws. Carter, however, found no mucous patches in 231 American Negroes with syphilis, although he found 21 cases among an equal number of white patients. (2) The rarity of tabes and paresis in yaws. These conditions are also rare in syphilis among primitive peoples in the tropics. (3) The reported absence of typical aortitis. Recent observations, however, show that aortitis is not uncommon, and Carl Weller (1936) from a study of the aortas of 169 patients in Haiti, a majority of whom had had yaws, concluded that yaws and syphilis produce identical aortic lesions. Hazen (1936) has emphasized the frequency of annular lesions and condylomas in the syphilis of the American Negro. This annular or circinate type of lesion is frequently noted in yaws (ringworm yaws).

The morphologic identity of the organisms, the common serologic reactions, the cross immunity, the response to the same therapy, as well as the general similarity of the experimental lesions and the clinical manifestations indicate that the diseases are, at least, very closely related.

Laboratory Diagnosis. In the primary and secondary stages the organisms may be found in large numbers in serum expressed from the lesions or in fluid aspirated from enlarged lymph nodes, preferably in fresh preparations, examination being made with darkfield illumination. After the third or fourth week the serologic reactions for syphilis become positive in over 80 per cent of the active cases.

Pinta (Carate)*

In the ninth edition of this manual pinta was described as a fungous infection, and note was made that Brumpt listed 27 different species of fungi as responsible: one for red pinta, one for blue pinta, and others for various shades down to the terminal white areas. This represents a sad chapter for medical research. In 1926, Menk reported that he had obtained positive Wassermann reactions in 74.5 per cent of the cases of pinta he had studied. Subsequently, Dr. Salvador Gonzales Herrejon, the great Mexican authority, rejected the mycologic origin, but was unable to find treponemas.

Our modern concept of the disease dates from August 1938, when Saenz, Triana, and Armenteros reported finding treponemas, indistinguishable from

*This section on "Pinta (Carate)" (pp. 172–175) was written by Rear Admiral E. R. Stitt.

Treponema pallidum, in the serum expressed from early papular lesions of plantar hyperkeratoses. These findings, in Havana, Cuba, were soon confirmed by Herrejon, in Mexico. Brumpt named the spirochete, discovered by Armenteros, *Treponema carateum.* (By priority this is the name for the parasite, and *T. herrejoni* is a synonym.) The failure of Herrejon to find treponemas was due to examining mature pintids (a name—analogous to syphilids—given the hyperchromic lesions by Leon-Blanco). It is in the early papules that *T. carateum* can most successfully be found (in practically 100 per cent of cases).

Clinical Manifestations. We now know that a papule develops within about three weeks, following experimental inoculation, and extends peripherally as a squamous erythematous patch, reaching a diameter of 1 cm. in about a month, but continuing to spread peripherally. Secondary lesions, in crops, appear around the primary lesion, or elsewhere on the body, in about five months (earlier or later). With progressive hyperpigmentation and, later on, depigmentation, there appear various-colored or vitiliginous-like spots, extending over the body, resembling the rarely seen syphilitic generalized leukomelanodermia.

In a series of 28 volunteers, including himself, Leon-Blanco selected several groups. One group included 17 healthy individuals having no gross or serologic evidence of syphilis or pinta. Another group was comprised of three syphilitics with positive serologic reactions. Five pinta patients with pintids, treponemas, and positive serologic reactions made up another group. All the volunteers were inoculated with pinta treponemas. All of the healthy volunteers, except three who were inoculated by serum deposited on intact skin, developed the primary papule, progressing to secondary papules. The three syphilitics developed the papule but the secondary eruptions were scant and atypical. Of the pinta volunteers, in one person there was a characteristic take, and in the other four no infection was manifest. Positive Wassermann reactions were not obtained prior to the development of secondary eruptions. In those with secondary eruptions about 60 per cent had positive reactions, and only in the advanced cases, with marked pigmentation, did the positive reactions approach 100 per cent. It would appear that immunity develops much more slowly than it does for yaws or syphilis. It is generally accepted that the immunity for yaws requires a longer period than that for syphilis.

Older descriptions of pintids gave various and changing colors: leaden, red, blue, yellow, violet, black, and white. In cases showing treponema origin, mercurial ointment-like spots are noted in the earlier lesions, becoming more pigmented and, later on, depigmented as final atrophy develops. It was observed years ago that the white-spot lesions never could be cured.

Fox noted resemblances between the plantar hyperkeratoses of patients in the clinic of Saenz in Havana, and those he had observed in Mexico, and this led to confirmation, in Mexico, of the connection between these plantar and palmar hyperkeratoses and pinta. In 1922, Moss and Bigelow, studying 579 patients with yaws in the Dominican Republic, found plantar hyperkeratoses to be the most common manifestation of yaws—327 of these patients showed plantar hyperkera-

tosis alone (crab yaws or clavus). Hudson reported hyperkeratoses and depigmentation in some of his cases of bejel in Syria.

Gutierez (1923), in the Philippines, reported 431 cases of keratosis palmaris and plantaris in 658 patients in his yaws clinic. The illustrations and descriptions noted in these cases from the West Indies, Mesopotamia, and the Orient seem similar, whether regarded as associated with syphilis, yaws, or pinta. Hasselmann, in a very short survey of nonvenereal bejel, claimed that biopsies of bejel lesions showed the disease to be syphilis, and disputed the statements of Hudson, who had had years of experience with bejel, that it was a disease innocently acquired and largely an infection of children.

Levaditi-stained sections of yaws and pinta show the spirochetes chiefly in the acanthosis areas of the epidermis, or in the spaces separating the papillae of the corium from the interpapillary pegs of the epidermis. The major changes in the vessels of the corium, as noted in syphilis, are wanting. Most authorities stress the great infrequency of aortic lesions, especially aneurysm, and spinal-fluid changes in yaws. Saenz, in 30 cases of pinta, found that 23 per cent of the patients had aortitis or aneurysm, and 10 per cent had spinal-fluid changes. Saenz also noted juxta-articular nodes in one of his patients.

Epidemiology. Epidemiologically, a vector is not required, although *Hippelates pallipes,* with yaws, and *Simulium haematopotum,* with pinta, have been incriminated. Leon-Blanco demonstrated the causative treponema in the discharges from fissures in the plantar hyperkeratoses of 29 of 41 patients. This highly infectious discharge could inoculate any abraded surface.

Pinta is favored by a moist warm climate, such as prevails along the banks of streams in the valleys of tropical or subtropical countries. The disease occurs especially with isotherms of 80° F., and above. Moisture seems more important for the persistence of both yaws and pinta, in the Tropics, than any other factor. Indeed, yaws almost disappeared in Jamaica during a record drought, from 1838 to 1848.

Treatment. The treatment of pinta is the same as for yaws and syphilis. The relationship of these three diseases is a challenge to medical science and, at the same time, of immense practical importance. Unfortunately, this problem is as buttressed by prejudice as is any theologic controversy.

Pinta-Syphilis Relationship. Guillermo Tellez (1889) discussed pinta as an infection of syphilitic nature, noting that the skin lesions simulated an exanthematic syphilis. Another authority on pinta, Gastambide (1881), notes that women may contract the disease through sexual intercourse.

The most interesting study of the development of pinta has been that of Aguirre Pequeño (1943–1944). Pequeño inoculated himself with pinta material in 1939 and at intervals reported the progress of the infection. The clinical manifestations were observed by his medical colleagues. Erythematosquamous spots appeared on the seventh day at each of four inoculations. These Pequeño called empienes. The period of dissemination began four and one-half months later, the 120 spots being similar to the initial ones. The spots on the gluteal regions resembled leprosy and those of the backs of the hands resembled pellagra. In the third or dyschromic

stage Pequeño noted that the vitiligo of the backs of his hands and the lower third of his legs was characteristic for pinta. During the development of the disease he reported, at various intervals during three and one-half years, mucocutaneous lesions of the genital area. Alopecia was also a feature. The palms of his hands and soles of his feet showed hyperkeratoses. Finally he was tortured by lancinating, fulgurating, unbearable girdle pains, which demanded relief from his martyrdom. Following penicillin (1,200,000 units) all manifestations of pinta disappeared but the locomotor ataxia-like pains persisted. These finally yielded to mapharsen and sodium iodide therapy. Pequeño has given us elsewhere one of the best outlines of the history of the disease.

CHAPTER 7

Rickettsia and Bartonella*

Rickettsiae

Diseases Due to Rickettsiae

The rickettsial diseases are a group of illnesses that occur throughout the world. They are caused by the rickettsiae, tiny microörganisms midway between the bacteria and the filtrable viruses, requiring living cells for proliferation, staining poorly with ordinary bacteriologic stains, and for the most part transmitted by arthropods. The microörganisms are named after Ricketts who first saw and described them in cases of Rocky Mountain spotted fever in 1908. Subsequently, in 1910, Ricketts noted similar organisms in body lice infected with typhus fever.

The rickettsiae and the diseases they cause can be classified by several different schemes. By some the diseases are spoken of as the typhus fevers and are classified according to the transmitting vector, i.e., louse typhus, tick typhus, mite typhus, etc. This classification, however, proves to be inadequate when more than one rickettsia can be transmitted by the same vector or when applied to Q fever which apparently can be contracted in the absence of known arthropod vectors. A more satisfactory classification is one based upon the etiologic agent per se and its antigenic relationship to others in the group.

There are six groups of human rickettsial diseases: typhus fever, Rocky Mountain spotted fever, boutonneuse fever, tsutsugamushi disease, Q fever, and miscellaneous diseases as yet not sufficiently understood for accurate classification.

Typhus Fever: Epidemic Typhus Fever. Epidemic typhus fever is a louse-borne disease caused by *Rickettsia prowazeki*. As it occurs classically it is a disease of winter, of populations going through famine or war, of populations heavily infested with body lice. To this group belongs the typhus fever that appears throughout the highlands of Mexico and of Central and South America. It is found in North China, the Balkans, Poland, and Russia in Europe, and throughout North Africa and the Middle East. The fatality rates vary from time to time; when the disease appears more or less sporadically it may be as low as 8 to 10 per cent; during serious major epidemics the rate has been as high as 70 to 80 per cent.

Murine (Endemic) Typhus Fever. This milder type of typhus fever is transmitted to man from infected rats by fleas (Dyer et al., 1931) and is caused by *R. mooseri*. It is a disease of summer and fall, the seasons when the fleas (*Xenopsylla cheopis*) are most prevalent. The disease can usually be traced to the place of work rather than to the place of residence (Maxcy, 1926). At the place of work,

*This chapter was contributed to in part by Dr. Paul W. Clough.

those with the greatest exposure to rats and their harborages, such as employees of feed and grain stores, groceries, and restaurants, suffer the highest attack rates. Murine typhus has been reported as occurring throughout the entire civilized world, particularly in seaports. The fatality rate is quite low, usually less than 5 per cent.

Rocky Mountain Spotted Fever. This is a moderately severe disease transmitted to man by a tick and caused by *R. rickettsii*. It is a disease of spring and summer, the seasons when the ticks are most prevalent. For years the disease was thought to be limited to the Rocky Mountain area of the United States, the habitat of the wood tick, *Dermacentor andersoni*. In 1931 (Badger, Dyer, and Rumreich) it was found to be prevalent in the eastern part of the United States, the area of the eastern dog tick, *Dermacentor variabilis*. Since 1930 the disease has been recognized in Brazil (São Paulo exanthematic typhus, Minas Geraes fever) and Colombia (Tobia fever, Colombian spotted fever), and more recently in Mexico. In the United States it has now been reported from 42 of the 48 states. The crude fatality rate for the entire United States is about 20 per cent, being 12 per cent in the age group under 15 years, and more than 40 per cent in the age group over 40 years (Topping, 1941). An infected female tick is able to transmit the disease to her offspring through the egg, so that the infection can be kept alive without another reservoir of virus. It has been shown recently, however, that dogs in infected areas in the east have complement-fixing antibodies in their blood and may play a part in the epidemiologic spread of the disease (Shepard and Topping, 1946).

Boutonneuse Fever (*Fièvre Escaire Nodulaire*). This is a relatively mild disease transmitted to man by a tick (*Rhipicephalus sanguineus*) and caused by *Rickettsia conori*. This rickettsia is antigenically related to but not identical with *R. rickettsii* (Gordon and Parker, 1943). The disease is known to occur around the Mediterranean and differs clinically from Rocky Mountain spotted fever in that it is a milder disease and that it has a primary eschar at the site of the infecting tick bite. Although not definitely proved, the disease known as South African tick bite fever is probably identical with boutonneuse fever (Gear, 1938).

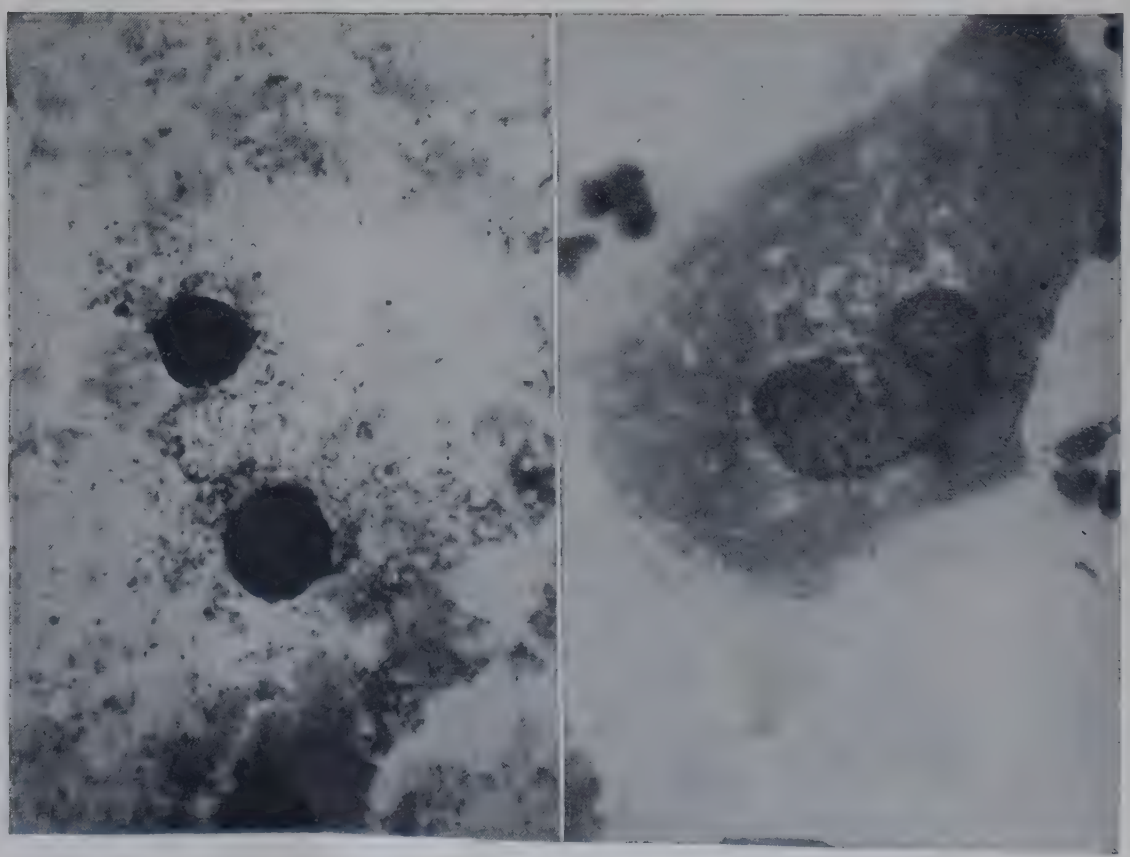
Tsutsugamushi Disease (*Scrub Typhus*). This disease is manifest by a moderately severe illness transmitted to man by a mite (*Trombicula akamushi, Trombicula deliensis,* etc.) and caused by *R. orientalis*. It was originally described in Japan as river fever, since it occurred following the floods. It has now been reported from most of the islands of the south and southwest Pacific; the East Indies and the Malay Peninsula; and Korea, Indo-China, Burma, and Assam on the mainland of Asia (Lewthwaite and Savoor, 1940). Since the transmitting mite feeds on animals or man only once during the larval stage and at no other period in its life cycle, it is probable that the adult female transmits the infection to her offspring through the egg. This disease, like boutonneuse fever, is characterized by a local eschar at the site of the infecting arthropod bite. Fatality rates in tsutsugamushi have varied from less than 5 per cent in some localities to over 30 per cent in others (Blake et al., 1945).

"Q" Fever. This is a new member of the rickettsial group which has been recognized only since 1937. It was first described as a relatively mild febrile illness that occurred in abattoir workers in Australia (Derrick, 1937) and caused by *R. burneti* (Burnet and Freeman, 1937). Although the causative agent has been isolated from ticks both in the United States (Davis and Cox, 1938) and Australia, human cases have been reported without a history of arthropod bite. It is now known that the more serious cases are characterized by a pneumonitis (Hornibrook and Nelson, 1940) and several rather extensive outbreaks have occurred.

Miscellaneous. There are several diseases apparently caused by rickettsiae—Indian tick typhus, trench fever, etc.—about which knowledge is insufficient for accurate classification.

Rickettsia Prowazeki

Rickettsia prowazeki, the cause of epidemic typhus, is the type species of the genus. Although this organism can be demonstrated in infected animal tissues and in the gut of infected lice, it is easily seen in preparations from the yolk sacs of hens' infected eggs, where under proper conditions the rickettsiae grow well (Cox, 1938). Smears are made by tearing off a small piece of tissue from the yolk-sac membrane, wiping off most of the yellow yolk on glass, and then macerating the tiny bit of tissue on an ordinary glass slide. The smear may be stained by Machiavelli's method (the rickettsiae usually stain red) or with Giemsa stain (the rickettsiae stain a pinkish purple). The organisms show considerable varia-



Rickettsiae from endemic typhus. (*Left*) Smear of gut of flea (*X. cheopis*). (*Right*) Scraping of guinea pig tunica showing intracellular and extracellular rickettsiae. Giemsa stain. (× 945.) (Courtesy, Dr. R. E. Dyer, National Institute of Health.)

tion in size, from almost invisible particles to rods which may measure more than 2μ in length. Diplococcoid forms are frequently seen particularly in preparations of *R. orientalis*.

The rickettsiae have never been grown except in the presence of living cells. *R. burneti* has been shown to pass the bacterial filters consistently, and under certain conditions so will *R. prowazeki*. Most of the rickettsiae can be sedimented by centrifugation in the small angle centrifuges at about 3500 to 4000 r.p.m. A soluble antigen (probably protein in nature) has been described as being released from *R. prowazeki*, *R. mooseri*, and *R. rickettsii*, upon exposure to diethyl ether.[1, 2] This soluble antigen is capable of eliciting all the immunologic responses that are seen with the intact organisms. By means of the electron microscope it appears that some of the rickettsiae (*R. prowazeki* and *R. mooseri*) are enveloped by a "capsular substance"[3] which may be the source of the soluble antigen.

A variety of ordinary laboratory animals are susceptible to infection with the rickettsiae. The guinea pig is probably the animal most universally used for investigations. It is susceptible to intraperitoneal infections with all the rickettsiae. A febrile illness following an incubation period of usually three to six days is observed, and with *R. mooseri* and *R. rickettsii* typical scrotal reactions can be seen. When *R. prowazeki*[4] and *R. mooseri* (Gildermeister and Haagen, 1940) are growing well in the yolk sacs of fertile eggs, a suspension of living organisms is toxic for white mice when inoculated either intravenously or intraperitoneally. Death occurs usually in two to six hours following the injection of the rich, living suspension of either organism. White mice are also satisfactory in investigations involving *R. orientalis*. Depending upon the dilution of the inoculum, death occurs usually in from 5 to 20 days following intraperitoneal inoculations. Some yolk sacs infected with *R. orientalis* when diluted with sterile skimmed milk to 10^{-8} or 10^{-9} have caused deaths in white mice. These animals can also be used for investigations of Q fever, and even though death does not ensue, some 7 to 10 days following inoculation they often have enlarged spleens and the rickettsiae can be demonstrated. Monkeys are quite susceptible to infections with *R. rickettsii* and have also been used in studies of the other rickettsial agents. A wide variety of rodents, both domesticated and wild, have been shown to be useful for various investigative purposes in studies of the rickettsiae (Brigham, 1937; Gear and Davis, 1942; and Snyder and Anderson, 1942).

Methods have been described for the preparation of antigens from the rickettsial agents that can be used in a variety of serologic tests. The complement-fixation test is probably the most widely used laboratory aid in the diagnosis of the specific rickettsial infections. Rickettsial agglutination tests have yielded useful information as has the precipitin test.

[1]Topping, N. H., and M. J. Shear: Studies of antigens in infected yolk sacs. (Approved for publication March 19, 1942; assigned date of publication in *Pub. Health Rep.*, March 27, 1942; withheld for security reasons.) *Pub. Health Rep.*, 59:1671–1675, (Dec. 29) 1944. Also published in "Studies of Typhus Fever," National Institute of Health Bulletin No. 183, Gov. Print. Off., 1945, p. 13.

[2]Topping, N. H., and C. C. Shepard: The preparation of antigens from yolk sacs with rickettsiae, *Pub. Health Rep.* (in press).

[3]Shepard, C. C., and R. W. G. Wyckoff: The nature of the soluble antigen from typhus rickettsiae, *Pub. Health Rep.* (in press).

[4]Bengtson, I. A., N. H. Topping, and R. G. Henderson: Epidemic typhus: Demonstrations of a substance lethal for mice in the yolk sac of eggs infected with Rickettsia prowazeki. (Approved for publication July 2, 1942; assigned date of publication in *Pub. Health Rep.*, July 31, 1942; withheld for security reasons.) "Studies of Typhus Fever," National Institute of Health Bulletin No. 183, Gov. Print. Off., 1945, p. 25.



A field in a more dilute Wilmington typhus preparation. This suspension was from infected mouse lung. (× 18,000.) (Courtesy, Shepard and Wyckoff: Public Health Reports, 61: No. 22, May 1946.)

Neutralization tests have been worked out for most of the agents and have been used to determine the neutralizing titers of serum from either convalescents or those vaccinated.

Laboratory Diagnosis of Rickettsial Diseases

Many of the procedures involved in the laboratory diagnosis of the rickettsial diseases carry with them the danger of accidental infection of personnel. This may be modified, however, by the vaccination of personnel, particularly against Rocky Mountain spotted fever and epidemic and murine typhus. We may assume that the rickettsiae are highly infectious, and accumulated laboratory experience indicates the danger from air-borne infection without apparent laboratory accidents as well as from breaks in technic.

The laboratory diagnosis of the rickettsial diseases depends in general upon two forms of tests: (1) the demonstration of a rise in antibody, and (2) the isolation and identification of the causative agent.

Immunologic Reactions: Weil-Felix Agglutination Test. With a rickettsial infection several types of antibody show a marked rise for varying lengths of time. The Weil-Felix reaction (agglutination of one of the proteus X strains) is one of the simplest tests that can be performed in the laboratory for confirmation of the clinical diagnosis. Since the demonstration of a significant rise in agglutinin titer

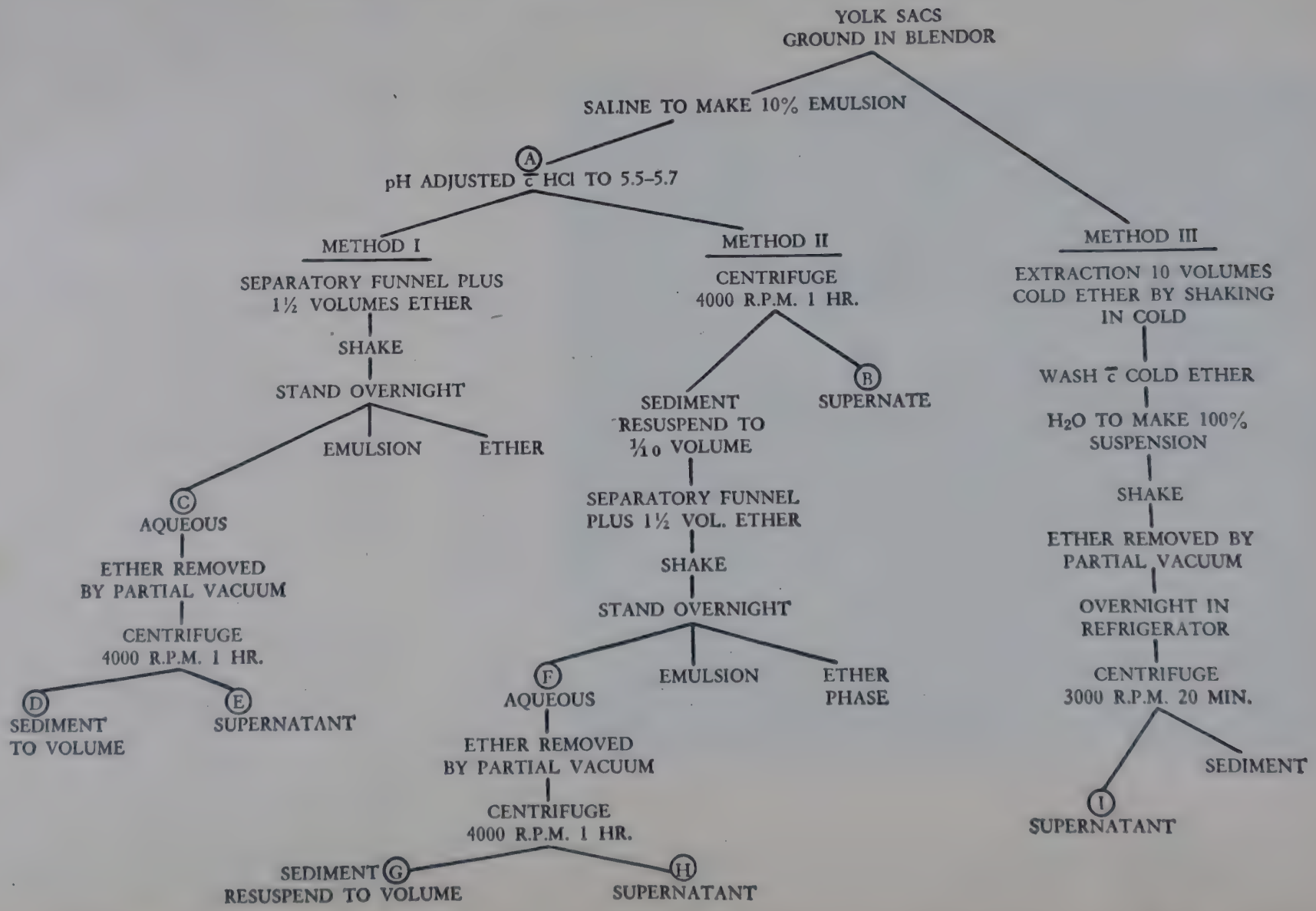


A field in another Breinl typhus preparation extracted with warm ether. Many free droplets of soluble antigen are distributed over the substrate. (× 18,000.) (Courtesy, Shepard and Wyckoff: Public Health Reports, 61: No. 22, May 1946.)

is of prime importance, it is well to have an early specimen and several others at five- to seven-day intervals. Agglutinins for the proteus X strains usually appear late in the second week of the disease, reach a peak titer, and disappear fairly early. Rocky Mountain spotted fever, epidemic typhus, and murine typhus usually produce agglutinins for proteus OX_{19}; while tsutsugamushi disease causes a rise in titer only for proteus OXK. The Weil-Felix reaction is of no value in differentiating between the Rocky Mountain spotted fever group and that of the typhus fevers. Q fever does not agglutinate any of the known proteus X strains.

In performing the test one must be careful to have a nonmotile strain of either X_{19} or XK. Variants in both occur, quite commonly in the latter, so that rather frequent observations of the cultures are advisable. Although there has been some controversy over living antigen as compared to alcohol, phenol, or formalin preserved, any of the four seem satisfactory. Usually the test itself is done macroscopically using water-bath incubation at 37° C. for two hours and refrigeration overnight. The microscopic (rapid-slide) agglutination test is used in some laboratories and when properly performed is satisfactory. Recently, a suspension of proteus OX_{19} containing a dye (methylene blue) has been advocated as an aid

DIETHYL ETHER TREATMENT OF YOLK-SAC MATERIAL



to the bedside diagnosis (Castaneda and Silva, 1938). In this test a drop of whole blood is mixed with the stained suspension and gently agitated either on a glass slide or prepared paper. The agglutination of the stained organisms can be readily seen with the naked eye.

The chief advantage of the Weil-Felix technic is that it can be done in any laboratory where agglutination tests are performed; however, it has many limitations and disadvantages which are causing it gradually to be superseded by other more specific technics.

Complement-fixation Tests. Complement-fixation tests (Bengtson and Topping, 1942) for the diagnosis of rickettsial infections are being used routinely in many

Table 13

Results of Diethyl Ether Treatment of Yolk-sac Material by Complement-fixation. End-point Titers*

Rickettsiae	METHOD I					METHOD II			METHOD III
	Crude 10% Emulsion		℞ *10% Saline Emulsion*			℞ *Sediment 10 × Conc.*			
	Whole	*Supernatant*	*Aqueous*	*Sediment*	*Supernatant*	*Aqueous*	*Sediment*	*Supernatant*	*Cold Ether Extraction*
Flow Chart Designation	A	B	C	D	E	F	G	H	I
R. prowazeki	1:32	1:16	1:512	1:4	1:256	† >1:2048	1:256	1:2048	>1:2048
R. mooseri	1:64	1:8	1:256	1:8	1:256	1:2048	1:256	1:2048	>1:2048
R. rickettsii	1:2	1:1	1:4	0	1:4	1:32	1:4	1:32	1:32
R. burneti	1:8	0	1:4	1:4	0	1:32	1:32	0	1:4
R. orientalis	1:8	1:4	1:4	0	1:2	1:2	0	1:1	1:128

*Only 3+ and 4+ reactions.

†>No end-point at given titer.

laboratories at present. Since these tests use specific rickettsial antigens, they can be used to differentiate between infections caused by different species of rickettsiae. Further, the antibody reacting in the complement-fixation test usually persists for long periods, and therefore positive reactions may be obtained years after actual infection. Most of the antigens used have been obtained by processing yolk sacs infected with the rickettsiae. The preceding chart outlines three methods for the preparation of antigens and Table 13 compares the results when the three methods are applied to five of the rickettsiae (Topping and Shepard, *loc. cit.*).

The actual technic of the test varies but little from that described in all standard procedures (Bengtson, 1944). Usually two to four units of antigen and antibody are used and two units of complement. Fixation may be done either at icebox temperatures or at 37° C. The test is read the following morning and the amount of hemolysis that has oc-

curred is recorded. There is considerable crossing by some of the agents, but by simple dilution alone it is usually possible to tell one from the other. Absorption technics can be resorted to if more quantitative results are desired. In reversing the usual procedure and testing for antigen in the presence of specific antibody, the test is of great assistance in studying the various antigenic fractions that can be prepared by chemical methods.

Precipitin Test. By chemical methods an antigen (soluble) has been prepared from epidemic and murine typhus which is suitable for a precipitin test.[5] By using the optimal-proportions method for determining the proper amounts of antigen and antibody as well as by absorption technics, quantitative results may be obtained. This test is of assistance in determining any minor variations present in otherwise similar strains. The precipitin test is also of value in studying various antigenic fractions as well as the action of certain physical and chemical agents upon typhus antigens.[6]

Specific Agglutination Tests. The rickettsial agglutination test has been in use in several laboratories for some time (Zinsser and Castaneda, 1932; Fitzpatrick and Hampel, 1941; Van Rooyen and Bearcroft, 1943). It is dependent upon securing large numbers of rickettsiae in a relatively pure suspension. Owing to the small size of the individual organisms, the agglutinated clumps may offer difficulty in reading, particularly if there is much extraneous material present with a tendency to spontaneous settling. Like the precipitin test, the rickettsial agglutination test lends itself well to absorption technics where more quantitative results can be obtained.

Neutralization Tests. Virus neutralization tests for the presence of neutralizing antibody have long been in use for the identification of rickettsial infections. Suitable dilutions of the serum under test are allowed to incubate (usually at room temperature) with known infectious material and then inoculated into susceptible animals. Controls are inoculated with mixtures of the infectious material plus dilutions of known negative serum. A rapid test for the presence of neutralizing antibody has been described for murine and epidemic typhus fever.[7] This test depends upon the serum under tests neutralizing the toxic substance which is responsible for the rapid death of white mice. Sera from either convalescents or those having received vaccine have the power to neutralize this toxic substance in relatively high dilutions for prolonged periods of time. This test has become the standard by which potencies of vaccine are judged in the United States; guinea pigs are vaccinated and the sera titrated for protective antibody. In vaccinated animals and human beings the neutralizing antibody seldom reaches the heights seen in early convalescence from actual infection, but it does approach the levels seen in individuals recovered from typhus fever some 6 to 12 months previously. In vaccinated individuals neutralizing antibody can be demonstrated after the complement-fixing antibody has disappeared.[8]

[5]Shepard, C. C., and N. H. Topping: Technic of a precipitin test for the study of typhus fever. (Approved for publication May 2, 1944; assigned *Pub. Health Rep.*, May 19, 1944; withheld for security reasons.) "Studies of Typhus Fever," National Institute of Health Bulletin No. 183, Gov. Print. Off., 1945, p. 87.

[6]Shepard, C. C.: Typhus fever: Antigens of the rickettsiae of typhus fever and the changes produced by heat. (Approved for publication May 27, 1944; assigned date of publication in *Pub. Health Rep.*, June 9, 1944; withheld for security reasons.) "Studies of Typhus Fever," National Institute of Health Bulletin No. 183, Gov. Print. Off., 1945, p. 93.

[7]Henderson, R. G., and N. H. Topping: Epidemic typhus fever. Neutralization of the toxic substance. (Approved for publication February 25, 1943; assigned date of publication in *Pub. Health Rep.*, March 19, 1943; withheld for security reasons.) "Studies of Typhus Fever," National Institute of Health Bulletin No. 183, Gov. Print. Off., 1945, p. 41.

[8]Topping, N. H., R. G. Henderson, and I. A. Bengtson: Epidemic typhus fever: Studies of epidemic typhus vaccine. (Approved for publication January 25, 1944; assigned date of publication in *Pub. Health Rep.*, February 18, 1944; withheld for security reasons.) "Studies of Typhus Fever," National Institute of Health Bulletin No. 183, Gov. Print. Off., 1945, p. 65.

Isolation and Identification. The etiologic agent of the rickettsial diseases circulates in the blood, at least early in the disease. By inoculating an adequate quantity of blood into a susceptible animal the agent can be isolated and studied. In the guinea pig inoculated intraperitoneally with from 3 to 5 ml. of blood drawn early in the course of the disease, isolations are possible in from 60 to 75 per cent of the cases of Rocky Mountain spotted fever and epidemic and murine typhus. There is an incubation period of from 2 to 10 days, depending upon the strain involved. For example, a strain of Rocky Mountain spotted fever causing severe infection in the guinea pig will have an incubation period of only two days whereas a "mild" strain may have a five- to seven-day incubation period. After the incubation period the guinea pig's temperature becomes elevated for a variable number of days, again depending upon the strain involved. Temperatures above 39.6° C. are usually considered fever in the guinea pig.

In several of these infections, male guinea pigs develop a scrotal reaction which may be of considerable importance in classifying the strain. In Rocky Mountain spotted fever the scrotal reaction usually develops after the guinea pig has had fever for four to seven days. Some animals will die before the scrotal lesions develop. The reaction is initiated by a very slight diffuse erythema, and at this stage the scrotum becomes stretched and assumes a shiny appearance. The following day a rash is seen over the scrotum and this may extend upward on to the groin. These macules become darker very rapidly, so that within 24 hours the skin of the scrotum appears purpuric where the dark purplish macules have coalesced. The superficial layers of the skin slough, leaving ulcers which may have more or less bizarre shapes. Healing begins and is complete in about five days, leaving permanent scars on the scrotum. The mild strains of spotted fever seldom produce these lesions.

A characteristic scrotal reaction is produced in male guinea pigs by a murine strain of typhus fever. This reaction is entirely different from that of Rocky Mountain spotted fever. In murine typhus the reaction is often seen on the first day of fever. Here the deeper structures are involved rather than the superficial skin. The tunica vaginalis becomes edematous with marked injection. The scrotum appears swollen, and it is no longer possible to replace the testicles through the external ring into the abdominal cavity. Except that the skin of the scrotum is tightly stretched and reddened, there are no superficial lesions. After two to three days the reaction subsides with no permanent evidence of the previous pathologic changes. There are no consistent characteristic scrotal reactions with an epidemic strain of typhus fever, Q fever, or tsutsugamushi disease.

During the first three or four days of fever the different strains can be passed to other guinea pigs by using 1 or 2 ml. of whole blood or a suspension of some of the organs, such as brain, liver, spleen, or the washings of the tunica vaginalis. Animals which have recovered are solidly immune to subsequent infection with a homologous strain, but not to the other rickettsiae. However, murine and epidemic typhus exhibit almost complete reciprocal cross immunity. One of the standard methods of identifying an unknown strain is to study the cross-immunity reactions with known strains.

White mice are easily infected by intraperitoneal injection of blood from the patient

with tsutsugamushi disease. If infected, their fur becomes ruffled some six to eight days later and they appear ill; by the tenth to twelfth day their abdomens become distended with a clear viscous fluid and death occurs usually from the twelfth to fifteenth day. Smears made from material obtained by lightly scraping the peritoneal surface, when stained with methylene blue, Giemsa, or Machiavelli will reveal the rickettsiae in the cells. A rickettsial pneumonia can be produced in white mice inoculated intranasally with *R. prowazeki, R. mooseri,* and *R. orientalis* (Castaneda, 1938; Durand and Sparrow, 1940; Fulton and Joyner, 1945) and their lungs used as a source from which to prepare rickettsial suspensions.

Cultivation

Since the rickettsiae have been cultivated only in the presence of living cells, various forms of tissue culture for these organisms have been described. The simplest and so far, at least, the most satisfactory method is cultivation in the yolk sacs of hens' fertile eggs. The infection can be initiated in yolk sacs by the inoculation of 0.5 ml. to 1.0 ml. of infectious material, usually blood from an infected guinea pig. The fertile eggs are incubated at 37° C. for from four to seven days before inoculation. After inoculation they are incubated at 35° C. The eggs are candled each day to determine the viability of the embryo. It frequently takes several passages in eggs before the embryos begin to die. Suitable yolk sacs for further passage are selected by microscopic examination of stained smears of the tissue. The seed for passage is prepared by thorough grinding of the infected yolk sacs, either by hand with alundum, or in a tissue blender. Sufficient diluent to make a 10 per cent tissue suspension is added either before or after grinding. If the intention is to preserve the seed virus, the diluent had best be sterile skimmed milk; then the seed can be frozen and stored at less than −30° C. If the seed is to be used immediately, 0.85 per cent saline is satisfactory as a diluent. The seed is usually diluted further (1 : 10 to 1 : 1000 or higher) just prior to inoculation into other fertile eggs.

Bartonella*

Bartonella Bacilliformis. This is a minute, rod-shaped organism which was first observed by Barton (1909) in red blood cells of a patient with Oroya fever. It stains readily, by Giemsa's method, as a reddish-blue rod 1 to 2μ long and 0.2 to 0.5μ wide. There may be a purplish-staining granule (chromatin?) near one end. There may be several organisms in a single red cell—in pairs, short chains, or irregularly distributed. V and Y forms occur. There are also rounded or pear-shaped granules from 0.3 to 1μ in diameter. The organisms also occur in the endothelial cells of the blood vessels, lymph nodes, spleen, liver, and intestines—both in the acute febrile stage of the infection and in the verruga lesions. Strong observed motility in darkfield preparations.

The organism can be grown in Noguchi's leptospira medium, in suitable tissue cultures, and in developing chick embryos. Cultures may be obtained from the blood of patients with Oroya fever, and from the verruga lesions. Monkeys are susceptible to subcutaneous injections of cultures from either source; verruga lesions develop, but very rarely is there extensive invasion of the red blood cells. Monkeys develop similar lesions after direct inoculation of material from human verruga nodules, but attempts to reproduce the syndrome of Oroya fever by inoculations of blood have been unsuccessful. The organisms can be recovered in cultures from the lesions produced in this animal. The usual laboratory animals are not susceptible. Carrion, a Peruvian medical student, died of Oroya fever after deliberately inoculating himself with blood from a verruga nodule.

*This section on "Bartonella" (pp. 186–188) was contributed by Paul W. Clough, M.D.

Organisms similar to *Bartonella bacilliformis* have been found in dogs (*B. canis*), rats (*B. muris*), and some other animals. In these animals the infection usually appears to be symptomless, but after splenectomy there may develop a fatal febrile anemia similar to that associated with Oroya fever. Verrucous lesions have not been observed.

Bartonelliasis. The disease in man occurs as two clinical types: a generalized form, Oroya fever, and a localized form, verruga peruana. The etiologic identity of the two syndromes was long questioned, but is now established.

Oroya Fever (Carrion's Disease). After an incubation period of two to three weeks the disease begins with malaise, irregular remittent fever, prostration, headache, pains in the bones and joints, and the rapid, sometimes fulminant development of a severe anemia which is hemolytic in type. The red cells may fall to 1,000,000 and the hemoglobin to 20 per cent within two to three weeks, even, it is said, within a few days. In proved cases the mortality is from 20 to 40 per cent, death usually occurring within two to three weeks. In patients who survive, the constitutional symptoms and the anemia gradually abate, but within a few weeks almost invariably the verrucous eruption appears.

The diagnosis in the severer cases can be made readily from blood films, as many of the red cells (even up to 90 per cent, according to Strong) contain the parasites. In milder cases, however, the organisms may be sparse. There are often many nucleated red cells of all types and many reticulocytes, so that the anemia is macrocytic. There is a neutrophilic leukocytosis. The bone marrow is hyperplastic. Blood cultures in suitable media are valuable, particularly in cases in which organisms are sparse. Agglutinins appear in the serum, but the titer is usually low, and their demonstration is of questionable value as a diagnostic procedure.

Verruga Peruana. This stage of the infection is characterized by the appearance of a cutaneous eruption which may be one of two types. Most often it takes the form of many small miliary nodules on the face, extensor surfaces of the extremities, and, frequently, the mucous membranes. These lesions appear as bright-red spots which develop into nodules and may attain the size of a pea. Less often there are large nodular lesions the size of a pigeon's egg, usually located about the joints. These frequently ulcerate and may bleed profusely, since the lesions of both types of eruption are extremely vascular—practically hemangiomas. After two to three months these lesions gradually disappear. The mortality is very low, although death may occur from hemorrhage or secondary infection. Verruga peruana may follow recovery from Oroya fever, but in many cases there is no preceding severe illness. It is thought, however, that the eruption is preceded by a generalized infection which was mild and unrecognized.

Epidemiology of Bartonelliasis. The disease is limited to mountain valleys in the Andes, chiefly in Peru, although it also occurs in Ecuador and Colombia. It is limited to altitudes ranging from 800 to 3000 meters, corresponding closely to the distribution of the probable vectors. In these restricted areas the infection seems to be practically universal, and strangers entering the areas at night are almost certain to contract the infection unless effectively protected from insect bites. The vectors are believed to be sand flies (*Phlebotomus verrucarum* and *P. noguchii*). The organisms have been isolated from these insects, but the crucial experiment of actual transfer to man by the bite of a sand fly apparently has not been carried out. Battistini (1931) and Hertig (1937), however, reported the transmission of the infection to monkeys by the bite of Phlebotomus.

Positive blood cultures have been obtained from apparently healthy individuals, some of whom had recovered from a previous attack of the disease, whereas others gave no such history. In one community 10 per cent of the individuals tested were found to be carriers. These observations indicate that human carriers probably constitute an important reservoir of infection.

Recovery from either type of the disease appears to confer a substantial degree of immunity from both. Attempts have been made to produce an active immunity by the administration of formolized vaccine, apparently with some success at least in ameliorating

the course of the subsequent infection. Of one group of 22 vaccinated individuals carefully studied and later exposed to the infection under natural conditions, Howe and Hertig (1943) subsequently obtained positive blood cultures from 12, all of whom showed some manifestations of a mild infection.

In Colombia the species of Phlebotomus believed to be vectors in Peru have not been found, and it seems probable that transmission there was effected by some other species. It is probable also that the infection was introduced by human carriers rather than by these insects. Little is known as to the capacity of other species of Phlebotomus to convey the infection. Species which bite man are widely distributed, however (they have been reported in Texas), and the possibility that they might bring about a much more widespread distribution of the infection must be considered (Weinman, 1946).

CHAPTER 8

Filtrable Viruses

The nature of the filtrable viruses and their relationship to ordinary bacteria, on the one hand, and to inanimate substances such as enzymes, on the other, is still controversial. It is impossible to formulate a satisfactory definition which will include all the objects or substances commonly regarded as viruses and exclude all others. It is possible, however, to enumerate certain characteristics which most students regard as fundamental and which serve as a working, if inadequate, definition. The viruses under suitable conditions will pass through filters which retain ordinary bacteria (although some of the smallest bacteria will pass through the coarser filters). They will grow and multiply only within the living cells of a suitable host (although this is also true of certain protozoa and the pathogenic rickettsiae, which are not filtrable and are not usually regarded as viruses). They are capable of indefinite multiplication under such conditions, and they show a tendency to undergo alterations or mutations which may markedly change their characteristics and make possible their adaptation to new hosts or to other changes in their environment. There is no evidence that viruses arise de novo, but only from previously existing virus.

The viruses constitute a highly heterogeneous group. The larger viruses, such as those of psittacosis and vaccinia, are organisms in the sense that they possess some structure analogous to that of bacteria, and may be regarded as the "midgets of the microbial world" (Rivers). In the case of the smaller viruses, such as that of tobacco mosaic disease, the ultimate virus particles are single, huge nucleoprotein molecules which are not combined into structural units and obviously differ fundamentally from everything which has hitherto been regarded as a "living organism." Whether such viruses may be regarded as "living" depends to a great extent upon the definition of the term.

General Properties

Filtrability. The viruses, on account of their minute size, are capable of passing through filters that are impervious to ordinary bacteria. (The technic of filtration and the various types of earthenware and porcelain filters are described on p. 917.) The passage of the virus through such a filter, however, depends not only upon its size, but also upon a number of physicochemical factors in the filter and in the virus suspension. The chemical composition and viscosity of the medium, the duration of filtration, the degree of pressure used, and the temperature at which filtration is carried out influence the result. The relationship of the electrical charge of the virus particles (which is influenced in turn by the pH of the medium in which they are suspended) to that of the filter is a very important factor. Such suspensions, moreover, contain extraneous protein material upon which

the virus may be adsorbed. Although filtration in this way affords a convenient method of obtaining the virus relatively pure and free from ordinary bacteria, it is impossible to draw precise conclusions as to its size from the porosity of these filters.

The ability to pass through such filters does not differentiate the viruses sharply from the bacteria, since some of the latter may also be filtrable. Certain of the smaller bacteria, such as *Dialister pneumosintes* and the organisms of pleuropneumonia and agalactia, will pass through the coarser grades of filters. The slender, flexible spirochetes may work their way through the pores, particularly when filtration is prolonged. Some species of bacteria have been shown to exist in a minute, filtrable form from which typical organisms may develop in subsequent cultures. These forms have been considered by some to be a phase in the life cycle of bacteria.

Ultrafiltration. Ultrafiltration with collodion filters has been utilized by Elford as a means of determining the size of various viruses. Filtration through these membranes is not affected by extraneous factors to the same extent as that through the earthenware types. By variations in the proportions of an acetone solution of collodion and an ether-alcohol mixture, membranes can be made of graded porosities. The technic of preparing them is complicated, but the membranes obtained with each of the different mixtures have a uniform permeability.

Purification of Virus Suspensions. Virus suspensions, whether obtained from the animal body or from virus tissue cultures, necessarily contain much extraneous cellular detritus and protein which is not entirely eliminated by filtration. These substances interfere seriously with the study of the viruses, and purification of such suspensions has been attempted by various methods. In some cases the virus may be adsorbed completely by the addition of a small amount of kaolin or animal charcoal or aluminum hydroxide. The virus may then be eluted by suspending the kaolin in a weak solution of ammonium hydroxide. The kaolin is removed, and the solution neutralized. These substances, however, also adsorb some protein, and the suspension is not strictly protein-free.

The most useful method of concentrating a virus suspension and obtaining it in relatively pure form is by differential centrifugation in an ultracentrifuge. These centrifuges can develop a force 200,000 times that of gravity, can bring about the sedimentation of particles or molecules with a molecular weight of 1,000,000, and can concentrate molecules with weights of 20,000 or more. Purification is not complete, however, and if there are particles or molecules of tissue protein of the same size as the virus, they will be sedimented with it. From the rate of sedimentation it is possible to calculate the size of the virus particles, if certain other properties (shape, density, etc.) are known.

Tobacco mosaic virus and a few others have been concentrated by salting out with chemical precipitants, but there is some alteration of the virus, and most viruses are inactivated by such procedures.

Size. Measurements of the viruses in earlier studies were made indirectly, since their size precludes the possibility of observing them by ordinary light. Particles smaller than 200mμ (one-half the shortest wave length of visible light) cannot be precisely delineated. By using the electron microscope, it is possible to obtain photographs showing the size, shape, and to a limited extent differences in density within much smaller objects, and in this way many of the viruses have been directly measured. Some technical difficulties are encountered, however. It is not possible by direct inspection to determine whether the particle represents the "pure" virus or a tissue particle to which the virus is adsorbed. Unless the virus is in a relatively pure state, it may be difficult to differentiate virus particles from other particles in the suspension. Also it is necessary to use dried films, in which an uncertain amount of distortion of the virus particles may occur. The figures obtained by direct measurement, however, usually agree reasonably well with the values calculated from data obtained by centrifugation, ultrafiltration, and diffusion experiments, although there are some discrepancies. The known viruses range in size from the virus of psittacosis, with a diameter of 275mμ and a weight (molecular) of 8500 million, to

that of foot-and-mouth disease, with a diameter of 10mμ and a molecular weight of 400,000. Tobacco mosaic virus, according to Stanley, has a molecular weight of 43,000,000 and has the shape of a slender rod, 250 $\times$ 15mμ. Most of the viruses, however, are known or assumed to be approximately spherical.

Cultivation. A number of viruses have been propagated in tissue cultures containing susceptible tissue cells. Some of the viruses seem to require a medium in which there is actual proliferation of cells, whereas others need only the presence of living cells. It may be necessary to provide tissue from a susceptible species of animal, and even a special tissue from such an animal may be required. A medium containing minced chick embryo, fresh plasma, and Tyrode's solution is suitable for many of the viruses. Many will grow on the chorioallantoic membrane of the developing chick. The tissue cells in these cultures may show inclusion bodies similar to those found in the animal body. As a rule, the virus causes no conspicuous change in the appearance of these cultures, and multiplication must be demonstrated by animal inoculation. None of the viruses has been grown in any lifeless medium.

Chemical Nature. This has been studied chiefly in the case of tobacco mosaic virus and other related viruses, and vaccinia virus, because these are substantially the only viruses which have been obtained in a relatively pure state in sufficient quantity to make accurate analyses possible. Stanley (1935) obtained the tobacco mosaic virus in crystalline form. It was found to be a nucleoprotein with a molecular weight of about 40 million. From hydrolysates of this protein, Stanley and associates isolated definite and constant amounts of 13 different amino acids, as well as 6 per cent of nucleic acid of the type which occurs in yeast. Material having the same composition was obtained, regardless of whether the virus was derived from tobacco or from other plants which are susceptible to it. On the other hand, certain variant strains regarded as mutations were found to contain relatively different amounts of certain of these amino acids or to have acquired an amino acid which was not present in the original strain. The virus resists heating to 90° C. It is regarded by some as a self-propagating enzyme rather than a living substance.

The elementary bodies of vaccinia have been shown, particularly by Rivers and associates, to have a more complex chemical structure. Several distinct antigens have been demonstrated. In addition to protein, these bodies have been shown to contain fat, thymonucleic acid, copper, riboflavin, and biotin. They therefore seem more comparable to the bacteria than to the simplest viruses.

Toxic Substances. It has been shown in the case of lymphogranuloma venereum (Rake and Jones, 1944) and influenza viruses (Henle, 1946) that the injection of huge doses of active virus into mice may be followed by the early death of the animal with symptoms of intoxication. Extensive degenerative changes were found in the organs, including the liver and spleen, which are not ordinarily invaded by the virus and in which no propagation of the virus could be demonstrated. It was not possible to separate the toxic from the infectious activity of the virus, although during inactivation of influenza virus infectivity may be lost before toxicity is destroyed. The toxic property was neutralized by homologous immune serum, in the case of influenza in high dilution, and mice could be protected by preliminary vaccination.

Inclusion Bodies. In many of the virus diseases peculiar structures called "inclusion bodies" may be seen within the cytoplasm or the nucleus of the injured cells. They are easily seen in sections stained with Giemsa's, Mann's, or Castaneda's stain, and their demonstration is of great diagnostic importance. Examples of cytoplasmic inclusions are the well-known Negri bodies in rabies, the Guarnieri bodies in the epithelial cells in the lesions of smallpox and vaccinia, the psittacosis bodies, and the Bollinger bodies of fowl pox. Acidophilic intranuclear inclusions are found in yellow fever, Rift-Valley fever, herpes zoster and febrilis, chickenpox, and a number of virus diseases of animals.

The nature of these bodies is still debated. Many believe that they are aggregations or "colonies" of the virus, whereas others consider them to be merely products of a cellular

reaction stimulated by the virus. The presence of the virus in some of these inclusions has been proved by animal inoculation, but it may be assumed that protein elements of the host enter into their composition.

Elementary Bodies. The Bollinger bodies of fowl pox, when properly stained, are seen to be filled with large numbers of minute coccoid bodies lying in an amorphous matrix and surrounded by a lipoid capsule. These so-called elementary bodies (or Borrel bodies) are believed to be actual virus particles. They are almost at the limit of visibility. Ledingham has shown that these Borrel bodies are specifically agglutinated by immune sera. The Paschen corpuscles of variola and vaccinia, and similar elementary bodies occurring in psittacosis, are also believed to represent the actual virus.

Viability. The viruses are apparently destroyed by various physical and chemical agents, such as heat, ultraviolet light, and various disinfectants, in much the same way as are bacteria. Some viruses, such as that of infectious hepatitis, resist heating to 60° C. Viruses are very resistant to intense cold and to desiccation, however, and may remain viable outside the body for long periods. Many viruses retain their viability for long periods in 50 per cent glycerin, whereas the vegetative forms of bacteria are killed within a short time. The best method of preserving most viruses consists of freezing, desiccating in vacuo, and storing in sealed tubes at a temperature of —76° C.

Adaptation. Many of the viruses are pathogenic for only a single species of animal, whereas other species are relatively immune. Some viruses, however, have a remarkable ability to adapt themselves to a different species, and with this biologic modification often show an alteration of their characteristics so that they no longer behave in the same way toward the original host. For example, the virus of smallpox, after adaptation to the calf, produces vaccinia and not smallpox when reinoculated into man, although the immunity produced protects from both diseases. The virus of yellow fever, which naturally is highly pathogenic for monkeys and causes extensive lesions in the liver and other viscera, after serial passages through mice by intracerebral inoculation tends to lose its viscerotropic properties and become largely neurotropic. Repeated passages through tissue cultures and chick embryos resulted in more marked alterations, so that a strain was obtained (17 D) which has relatively little pathogenicity, but has been used successfully on a vast scale for the immunization of human beings. Analogous changes have been observed in many viruses. Such changes are believed to be the result of mutations. If such a mutation occurs and environmental conditions are sufficiently favorable, it will outgrow and eventually replace the original strain which gave rise to it. In some cases such mutations occur with considerable regularity and can be reproduced practically at will. In others, it appears to have been the result of a biologic accident, and has not been reproducible. Thus it is stated that attempts to reproduce a strain of yellow fever virus similar to 17 D from other natural strains of virus have been unsuccessful.

Immunity. Recovery from a virus infection is followed by some immunity from reinfection. In the case of some infections, such as yellow fever, the degree of protection is marked and the duration probably life-long. In others, such as dengue, the common cold, and influenza, the protection is brief and relatively slight. After recovery the serum acquires the power of neutralizing the virus. Such serum will protect animals if injected along with or just prior to inoculation of the homologous virus, but has little or no effect after the development of symptoms. After virus has once entered the cells, it seems to be protected from the action of antibodies in the serum of the host. The immunity is specific for the particular virus concerned, and in some cases (as foot-and-mouth disease and influenza) for the particular type of the virus. Such sera may show specific agglutinating or precipitating and complement-fixing properties if suitable preparations of antigen are available for such tests.

The reasons for such marked differences in immunity are not definitely established. It has been suggested that life-long immunity is associated with persistence of virus within the body. There is some reason to believe that, at least in the case of some diseases, all

the cells that become infected acquire the infection during the initial stages of the disease. Once within the cells, the virus is protected from the antibodies in the serum, and may persist indefinitely although unable to reach the exterior and infect other hosts. In the case of influenza, on the other hand, the virus invades the superficial cells of the respiratory passages. These are rapidly desquamated, and the virus is eliminated with them, and does not exert a continuing stimulus to antibody production. Herpes virus, on the other hand, persists in the body for years, and is almost continuously discharged, in spite of high antibody content in the serum.

Neutralization of virus by antibody does not necessarily involve actual destruction of the virus. Under certain conditions, infectious virus can be recovered from neutral mixtures by cataphoresis or even by simple dilution with salt solution. Some believe that the serum does not act directly on the virus, but on the tissue cells in such a way as to protect them from invasion.

Interference. Infection of an animal with one virus may prevent the establishment of an infection with another unrelated virus. This has been observed, e.g., in the case of yellow fever and Rift-Valley fever viruses, poliomyelitis and lymphocytic choriomeningitis, and equine encephalomyelitis and St. Louis encephalitis. It has been observed in chick embryos with different strains of influenza. In monkeys, inoculation with a neurotropic strain of the virus of yellow fever may afford protection from an otherwise fatal inoculation of a "viscerotropic" strain. This is not due to the development of an ordinary immunity. The first virus seems to preoccupy some chemical constituents of the cell which are essential for the propagation of the second virus or possibly in some way it prevents penetration of the cell by the second virus.

Vaccination. Vaccination with a living virus may produce a high grade of immunity, but it is necessary to use a virus which has been attenuated in some way without loss of its antigenic properties. This can sometimes be accomplished by repeated passage of the virus in another species of animal. Neutralization of the virus by homologous antiviral serum, and attenuation by drying and by various chemicals have also been employed. Carbolized or formolized vaccines have been used extensively in veterinary work.

In vaccination with living viruses there is a potential danger of an unexpected increase in virulence, and only long experience with a given procedure can demonstrate its safety conclusively. This is also true of viruses rendered inert by chemicals. It has been reported that a virulent virus has been obtained by cataphoresis from a formolized, apparently dead, suspension.

Early attempts to secure immunity by use of completely inactivated virus vaccines were largely unsuccessful, partly at least because the doses were inadequate. It has been shown that if adequate doses of killed vaccine are given, a substantial immunity can be produced from influenza, rabies, equine encephalomyelitis, and Russian spring-summer encephalitis, and it seems likely that this will prove true of other viruses.

Diseases Due to Filtrable Viruses

The filtrable viruses cause many of the diseases of plants, arthropods, fishes, birds, mammals, and man, and also of bacteria, if d'Herelle's concept of bacteriophage is correct. Some of the virus diseases (yellow fever, dengue, and phlebotomus fever) in man, and many in plants are transmitted by various species of arthropods. The transmission may be simply mechanical, or actual propagation in the arthropod may take place. In the latter case an "extrinsic incubation period" is necessary before an arthropod becomes infective, but, once infective, it remains so throughout its life. Findlay has suggested that some of these viruses may have originated as arthropod parasites and subsequently have become partially adapted to various species of vertebrate hosts.

Some of the viruses stimulate cellular proliferation (common warts, various fowl tumors, etc.) whereas others produce necrosis (yellow fever, Rift-Valley fever). Some of the viruses, such as that of foot-and-mouth disease, show a marked selectivity for one particular type of cell, whereas others parasitize the cells of various tissues.

Classification. Attempts have been made to classify the viruses by their affinities for cells derived from the different embryonic layers. These affinities, however, may vary in different species of animals, and some of the viruses isolated from one type of tissue may be adapted artificially to another type. There is, nevertheless, a fairly well-defined group affecting only cells derived from the ectoderm. In this group are the viruses of the common wart, molluscum contagiosum, and trachoma, which parasitize exclusively the cells of the skin or mucous membrane (the *dermotropic viruses*); the viruses of variola, vaccinia, varicella, herpes zoster, and febrilis, which may affect the nervous system as well as the skin; and the viruses of rabies, epidemic encephalitis (St. Louis type), poliomyelitis, and certain diseases of animals which affect the central nervous system (the neurotropic viruses). The viruses of Rift-Valley fever and yellow fever may parasitize cells from all the embryonic layers, and from these viruses variant strains having a particular predilection for the central nervous system have been produced. The influenza viruses are practically restricted to the epithelium of the respiratory tract.

Laboratory Diagnosis of the Virus Diseases. The presence of a filtrable virus in the body may be determined by the inoculation of blood, tissue, or other material into animals belonging to a species known to be susceptible to the suspected virus. (See Table 2, p. 22, for a list of suitable animals and methods of inoculation.) Some of the viruses are pathogenic only for monkeys, and others have been transmitted only to human volunteers. The symptoms and pathologic lesions in the inoculated animals may be sufficiently distinctive to identify the virus, and the identification is confirmed if control animals receiving a known virus-neutralizing serum in conjunction with the unknown virus are protected. The demonstration of cytoplasmic or intranuclear inclusion bodies in smears or sections from the inoculated animals is also of importance in the diagnosis of the diseases in which they occur. In rabies the demonstration of the Negri bodies in the brain of the suspected dog as well as in that of inoculated animals is the most important diagnostic procedure.

Laboratory animals are subject to natural virus infections. Such a virus may be present in latent or masked form but be stimulated to produce an active infection in the animal by the manipulations involved in the inoculation of foreign material. It is therefore necessary to identify the virus obtained before valid conclusions can be drawn from such experiments.

The presence of virus-neutralizing antibodies in the serum of patients may be utilized for diagnosis. The serum to be tested is mixed with a suspension of the known virus, and varying amounts are injected into a series of susceptible animals. Controls with a known normal and known immune serum should be made. Complement-fixing, agglutinating, and precipitating antibodies have been found in the serum in a number of the virus diseases, and when suitable antigens are generally available, their demonstration should become a useful diagnostic procedure.

Hypersensitiveness to the proteins in virus-containing material from buboes, inoculated mouse brain, and culture has been demonstrated in lymphogranuloma inguinale, and intradermal tests are of great value in diagnosis.

Serum Treatment. Immune sera have been used successfully in prophylaxis in a number of the virus diseases. The results of treatment, on the contrary, are unsatisfactory, as might be expected from the pathogenesis of these infections.

Smallpox, Alastrim, and Vaccinia. These diseases are caused by closely related viruses which are filtrable under suitable conditions. The three viruses are closely related antigenically. Recovery from any one of the infections is followed by an immunity from all of them, and the serum contains virus-neutralizing, agglutinating, precipitating, and complement-fixing antibodies which are active against all.

SMALLPOX. Smallpox virus stimulates the formation in the infected cells of eosinophilic inclusion bodies, Guarnieri bodies, which are easily recognized microscopically. They are from 1 to 20μ in diameter, and contain many minute elementary (Paschen) bodies, about 0.25μ in size. The latter are believed to represent the virus particles. Similar inclusions are found in vaccinia and in alastrim. Monkeys are susceptible to inoculation with smallpox virus, but other animals usually are not.

The incubation period is 7 to 18 days, often 12. Diagnosis is based chiefly on the distribution of the focal eruption which begins the third to the fifth day. This is most abundant on the exposed and extensor surfaces, face, backs of hands, forearms, more on shoulders than abdomen, etc. The character of the individual lesions and the history, as to vaccination or immunity, may be very deceptive. The virus is present not only in the skin but also in the nasal and buccal secretions, and the disease is communicable before the vesicular rash appears.

The following inoculation tests may be of secondary but uncertain aid in diagnosis.

PAUL'S TEST. The contents of a vesicle or pustule are inoculated into the cornea of a rabbit. After from 48 to 72 hours the eyeball is enucleated, fixed in strong sublimated alcohol, and examined for the characteristic whitish papules. Typical Guarnieri bodies may be found in the corneal cells. Negative reactions are obtained in about half the cases.

EGG TEST. Inoculation from lesions to the chorioallantois of the developing chick embryo may help in early purpuric toxic eruptions before the focal eruption but loses valuable hours.

MC KINNON'S TEST. Material from a lesion of the patient is inoculated intradermally into a normal rabbit and into a previously vaccinated rabbit. If the material contains the virus of smallpox, a red swelling develops in the normal rabbit in about four days and disappears by the twelfth day. The vaccinated rabbit shows no reaction or only a red nodule that disappears rapidly.

ALASTRIM. This is a mild form of smallpox which is widely prevalent in certain tropical regions and has been introduced into countries in the temperate zone, including the United States and Great Britain. The mortality is less than 0.5 per cent. With rare exceptions it runs a mild course when acquired by unvaccinated individuals, and thus differs from the mild form of ordinary smallpox as seen in vaccinated persons, which may give rise to a severe form of the disease. Vaccination protects from alastrim. Large bodies of well-vaccinated marines of the U. S. Navy were perfectly protected in the midst of a Haitian epidemic.

VACCINIA. Vaccinia, it is generally agreed, is smallpox which has been permanently modified by passage through the calf, probably as the result of a mutation.

Certain strains of human variola virus have been "converted" into vaccinia by successive passages through monkeys, rabbits, and calves. Monkeys are susceptible. Rabbits are more susceptible to vaccinia than to variola virus and show a rather typical eruption when inoculated subcutaneously.

VACCINATION. The material for the vaccine is taken from the vesicles of inoculated calves not later than one week after inoculation. The most potent material is in the pulp at the base of the vesicle, not in the lymph that exudes from the vesicle. The pulp is ground up and mixed with an equal amount of glycerin. The calves are then autopsied to make sure that no other disease is present. The virus is later tested for tetanus bacilli, pus organisms, and the virus of foot-and-mouth disease. The test for tetanus is very important. Cultures are made anaerobically and incubated for at least seven days and checked by inoculating mice if necessary. If found free from any harmful organisms, the vaccine is then tested on rabbits for potency.

Rivers has cultivated vaccinia virus in a medium of minced chick embryo and Tyrode's solution which, when injected intradermally in man, produces immunity from calf lymph vaccine without scarring or marked systemic reaction. Goodpasture and Buddingh have developed a practical method of producing vaccine virus by inoculating the chorioallantoic membrane of the chick embryo.

TECHNIC OF VACCINATION. Leake considers the multiple-pressure or prick method (acupuncture) probably the best method of vaccination. This consists of a shallow, tangential pricking of the skin with a needle through a drop of the vaccine covering an area not greater than 3 mm. (⅛ inch) in diameter. There is little chance of accidental infection, and the eruption is typical. Acetone has been found more satisfactory than alcohol for cleansing without irritating the skin. The needle is not thrust into the skin, but is held parallel or tangent to it, the forefinger and middle finger of the right hand above the needle and the thumb below. The needle should point to the operator's left and across the arm so that the thumb of the operator does not hit the skin. With a perpendicular motion from the wrist the side of the needle is then pressed firmly and rapidly into the drop about 30 times in five seconds. The elasticity of the skin will pull a fraction of an inch of the epidermis over the point of the needle at each pressure so that the vaccine is carried into the deeper epithelium (cuboidal prickle-cell layer) where multiplication takes place most easily. The remaining vaccine can be wiped off with sterile gauze. No signs of bleeding will occur, and evidence of the punctures will fade out in less than six hours. Formerly, when only six pricks or punctures were advocated, the method was shown to give fewer "takes" than the scratch method. By the use of 30 pricks the percentage of takes has been made as high as in any other safe method. For primary vaccinations, where the mildest possible take is desired, and where other attempts will be made promptly if the first fails, the number of pricks may be reduced to 10 or even to one.

The important points in vaccination are (1) to keep the vaccine below freezing if possible, and (2) to make a small superficial insertion.

REACTIONS TO VACCINATION. Vaccination performed in a proper manner with a potent vaccine will result in a reaction which may be classified under one of the following forms:

1. *Primary reaction or vaccinia,* which indicates an absence of immunity from smallpox. In this the zone of redness, rather narrow from the third to the seventh day, begins to spread suddenly about seven days after vaccination, is broadest in 8 to 14 days, and then fades rapidly and disappears. The reaction is accompanied by vesicle formation.

2. *Accelerated or vaccinoid reaction,* which indicates partial immunity. In this

the broadest redness occurs and passes in three to seven days after vaccination. Vesicle formation occurs.

3. *Immediate reaction or reaction of immunity,* which indicates full protection from smallpox. In this the broadest redness occurs and passes in 8 to 72 hours after vaccination. A slight elevation of the skin can be felt by passing the finger lightly over the vaccinated area. Vesicle formation is usually absent. This reaction can be simulated by a sensitivity reaction with inert vaccine, but fully potent vaccine usually gives a sharper reaction. Immunity is indicated only if one knows the vaccine is fully potent. Fully potent vaccine gives at least 50 per cent vaccinoid reactions in persons who had a successful reaction or smallpox itself not less than 10 years previously.

Varicella. This disease is presumably caused by a filtrable virus, although actual filtration has not been demonstrated. Inoculations of both man and monkeys with bacteria-free fluid from the vesicles, however, have produced the disease. Acidophilic intranuclear inclusions similar to those found in variola can be seen in the affected cells.

The relationship of the virus to that of herpes zoster is still controversial. Clinical observations on the association between herpes zoster and outbreaks of chickenpox have suggested that the etiologic agents might be identical. Recent reports on the results of complement-fixation tests in the two conditions support this view, but it is not generally accepted at present.

Herpes Zoster. This is due to a virus which has not been shown to be filtrable. The disease has been transmitted experimentally from one individual to another, and by inoculation of human skin grafted onto the chorioallantoic membrane of chick embryos (Goodpasture). Intranuclear cell inclusions indistinguishable from those of varicella can be demonstrated.

Foot-and-mouth Disease. Foot-and-mouth disease is a highly contagious disease of cattle, pigs, sheep, and goats and is characterized by a vesicular eruption in the mouth and on the feet and udders. Occasionally man contracts the infection by contact with infected animals or by drinking raw milk containing the virus. It is of interest that the disease is the first infection in animals found to be caused by a filtrable virus (Loeffler and Frosch, 1898).

The virus is present in the vesicles and in the saliva, excreta, and milk. In the prevesicular stage it has been found in the blood. In some instances the virus has been demonstrated in animals some time after recovery from the disease.

The properties of the virus have been studied particularly in England and on the continent in recent years. It is the smallest virus known, the size being estimated at from 8 to 12mμ. It is easily destroyed by heat and does not withstand pasteurization. It is, however, resistant to desiccation, and for this reason, in part, epidemics among cattle are extremely difficult to control. The virus has been cultivated in a medium containing the lips, tongue, pads, or skin of embryo guinea pigs, but seems to require particular tissue from a susceptible species of animal (Maitland). Three types of the virus have been differentiated immunologically, O, A, and C, and the immunity developed in an animal after recovery from infection due to one of these types does not extend to other types.

Hyperimmune serum prepared with these types has been used for prophylaxis and for treatment in cattle. Vaccination with formolized virus or serum-virus mixtures produces an active immunity and has been used to a limited extent in Europe.

Common Wart (Verruca Vulgaris). The epithelial cells of the common wart have been shown to contain inclusion bodies, and Kingery and Wile have obtained a filtrable virus which appears to be the etiologic agent.

Herpes Febrilis (Simplex). In this infection a filtrable virus is constantly present in the local lesions and sometimes in other tissues, including the central nervous system. Intranuclear inclusion bodies can be demonstrated in the affected cells. The virus has been grown in suitable tissue cultures, and grows readily on the chorioallantoic membrane of the chick embryo. Inoculation of material containing the virus on the rabbit's cornea causes a severe keratitis, and with many virus strains an encephalitis. A few cases of human encephalitis due to herpes virus have been reported. Animals which recover are immune.

It has been shown, by Burnet among others, that infection is commonly acquired in early childhood, presumably from contaminated saliva of infected adults. It often appears as an acute apthous stomatitis. Neutralizing antibodies appear in the serum in high titer, but this does not eliminate the infection which persists indefinitely in the tissues, often, probably, throughout life, without causing appreciable damage to the host. Conditions are therefore almost ideal for the perpetuation of the virus, and it represents the most perfect known adaptation of a virus to the human host.

Epidemic Keratoconjunctivitis. This is a mild infectious disease which is conveyed from person to person by direct contact and is prone to appear in epidemic form in men who are working in close contact under unhygienic conditions—"ship-yard conjunctivitis." The initial manifestation is an acute conjunctivitis characterized by intense congestion, edema, and a scanty nonpurulent exudate, which in 50 to 85 per cent of the cases is followed after one to two weeks by a punctate keratitis in one or (successively) both eyes. Discrete areas of infiltration about 0.5 mm. in diameter appear just beneath Bowman's membrane, mainly in the central area of the cornea. Ulceration is very rare, but the infiltrates often persist for months, in some cases indefinitely, and cause substantial diminution but not total loss of vision. By intracerebral inoculation of material from the eyes of two human subjects, Saunders (1942, 1943) succeeded in establishing the infection in mice. The agent was filtrable through membranes with a pore diameter of 75mμ. Saunders reported reproducing the disease in one human volunteer and in two monkeys by inoculating the virus into the conjunctiva. The virus was neutralized by serum from convalescent patients.

Encephalitis. Encephalitis in man may be caused by many different agents. It may occur as a complication or unusual manifestation of a number of virus infections (such as measles, mumps, vaccinia, lymphogranuloma venereum) in which tissues other than those of the nervous system are primarily involved. In other infections the virus is fundamentally neurotropic, and manifestations of encephalitis constitute the usual clinical picture. Of these the well-established types occurring in the United States are the Economo and St. Louis types of encephalitis, and the Eastern and Western types of equine encephalomyelitis. Also important are the Venezuelan type of equine encephalomyelitis, the Japanese type B encephalitis, and the Russian spring-summer encephalitis. The West Nile virus and the Semliki Forest virus are related viruses isolated by Smithburn et al. in Africa, but the part they may play in human disease has not been established. These viruses are all distinguishable antigenically, but some are so closely related as to suggest that they are variants of a common ancestor.

Epidemic Encephalitis (Type A) of Economo, "Lethargic Encephalitis." This was first recognized after the influenza epidemic of 1918. The acute stage is charac-

terized by fever of varying degree, somnolence, occasionally by delirium, cranial-nerve paralyses, ophthalmoplegia, oculogyric crises, and many other manifestations of brain injury. It is frequently followed by psychic disturbances, mental deterioration, or a Parkinsonian syndrome. These sequelae may follow an acute attack closely, or after a symptomless period of months or years, and they pursue a slow but relentlessly progressive course as a "chronic encephalitis." Neither the etiologic agent nor the mode of transmission of this type is known.

Epidemic Encephalitis of the St. Louis Type. This disease occurred as an extensive epidemic in St. Louis and its vicinity in 1933 and again in 1937. The onset is usually abrupt with high fever, headache, vomiting, muscular rigidity and tremors, mental confusion, lethargy or delirium, and often paralyses, which, however, rarely involve the eye muscles. The mortality has been about 20 per cent, but recovery is usually complete, and the serious sequelae of the Economo type rarely if ever occur. The incidence and mortality increase with advancing age, and children are relatively spared. Autopsies in man and animals show perivascular accumulations of cells and degeneration of nerve cells in the brain and upper part of the cord.

In 1934, by intracranial inoculation of filtrates into rhesus monkeys, Muckenfuss, Armstrong, and Webster demonstrated a filtrable virus in the brain of subjects whose death was due to this type of encephalitis. Swiss white mice are also highly susceptible to intranasal as well as intracerebral inoculation, and the virus can be cultivated on the chorioallantoic membrane of the chick embryo. The virus has not been obtained from the spinal fluid, blood (of man), or nasopharyngeal washings. By filtration experiments Muckenfuss et al. estimated that the virus particles were 22 to 33mμ in diameter. Specific neutralizing antibodies appear in the serum of the convalescent patient, and a rising antibody titer is regarded as proof that the individual has been infected with this virus. This may be demonstrated by mouse-protection tests, or by inoculation of chick embryos with serum-virus mixtures. By this means Blattner and Heys (1945) demonstrated the occurrence of sporadic cases of the disease (in 12 of 66 cases of encephalitis carefully studied) in the St. Louis area between 1939 and 1944, indicating the presence of an endemic focus in this region.

epidemiology. In 1941 Blattner and Heys demonstrated the transmission of the virus to mice by the bite of the tick *Dermacentor variabilis*. The virus was transmitted through the eggs to the third generation of ticks. In 1939 and 1940 an extensive epidemic of encephalitis in man and horses occurred in the Yakima Valley, Washington, due to both the virus of St. Louis encephalitis and that of the Western type of equine encephalomyelitis. Hammon et al. (1941, 1943) isolated both types of virus from "wild" *Culex tarsalis* mosquitoes caught in this region. Both have also been demonstrated in trapped *Culex pipiens* mosquitoes. St. Louis virus has been transmitted experimentally by bites of infected mosquitoes of both these species as well as by species of Aëdes and Culiseta. *Culex pipiens* is very common in the St. Louis district and is probably an important vector in that region. There is no proof, however, that the virus can be maintained in these mosquitoes.

St. Louis encephalitis, as well as the types of encephalitis discussed in the following pages, is most prevalent in the summer and fall when the arthropod vectors are most numerous. The Economo type, on the other hand, is most frequent in the winter.

In order to determine *reservoir hosts,* Hammon et al. (1942) tested the neutralizing power of blood obtained from a large number of wild and domesticated mammals and birds and obtained positive results for both viruses (the St. Louis and the Western equine) in a large percentage of animals, particularly in the domesticated group. Most frequently positive were the duck, goose, pigeon, turkey, and chicken. Chickens are easily infected by subcutaneous injections of small amounts of either virus, as by bites of infected mosquitoes, but rarely show manifestations of infection. However, if blood is taken 16 to 64 hours after injection of the virus, it regularly causes encephalitis in mice after intracerebral injection, and protective antibodies develop later in the blood of the chicken. Hammon and associates therefore regard domestic fowl as an important reservoir of infection. Howitt (1942) obtained similar results in a study of these infections in California. This view also receives support from the work of Smith, Blattner, and Heys (1944, 1945), who isolated the St. Louis virus from chicken mites (*Dermanyssus gallinae*) from three areas near St. Louis during a nonepidemic period. They also demonstrated the congenital transmission of the virus in these mites.

This evidence indicates that both St. Louis encephalitis and Western equine encephalomyelitis are naturally diseases of birds (fowl) which are well adapted to the virus and suffer little from it. The vectors are biting arthropods, including Culex mosquitoes, and possibly chicken mites and ticks, of which the latter two might also be reservoirs of virus. Infection of man or the horse appears to be a biologic accident, but as these mammals have not become adapted to the virus they commonly suffer serious illness from the infection.

Equine Encephalomyelitis of the Western Type. This disease was first studied by Meyer (1930) in an epizootic among horses in California. He isolated the virus and demonstrated its pathogenicity for mice, rats, rabbits, monkeys, and particularly guinea pigs. Extensive epizootics have occurred in Minnesota, Washington, and other western localities. Sporadic cases have occurred in man, chiefly in farmers who had been in contact with diseased animals. A fatal accidental infection in a laboratory worker has been reported. The virus has been transmitted experimentally by mosquitoes (Aëdes, Culex, Culiseta), *Dermacentor andersoni,* and *Triatoma sanguisuga. Culex tarsalis, C. pipiens,* and *Culiseta inornata* have been found naturally infected, but *C. pipiens* did not transmit the virus. Clinically the disease in man resembles the St. Louis encephalitis.

Equine Encephalomyelitis of Eastern Type. This disease has occurred in epizootics in horses in widely scattered areas along the eastern seacoast of the United States. A severe epidemic in man occurred in New England in 1938. Feemster reported a study of 38 cases, all but one in children, of which 25 were fatal. The disease begins abruptly with high fever, vomiting, headache, dizziness, irritability or drowsiness, and there develop muscular rigidity, tremors, and sometimes paralyses, convulsions, and coma. There were serious sequelae in some patients who recovered. The virus was obtained from the brain in some fatal cases and marked protective power was demonstrated in the serum of four patients who recovered. It is believed that birds constitute the reservoir of virus. Many species have been infected experimentally, and the virus has been isolated under natural conditions in ring-necked pheasants and pigeons. The virus has been conveyed experimentally by bites of Aëdes mosquitoes, but with difficulty, and some other arthropod is thought to be the usual vector.

Japanese Type B Encephalitis. This occurs in Japan, eastern China, and Russia. Clinically it resembles St. Louis encephalitis, but has a higher mortality (about 60 per cent).

The virus will infect mice, monkeys, and sheep by intracerebral or intranasal inoculation. It is conveyed by mosquitoes. The disease has not been recognized in the United States, but neutralizing antibodies have been demonstrated in the serum of some individuals in this country.

Russian Spring-Summer Encephalitis. This is caused by a virus which is closely related antigenically to the virus of the louping ill of sheep observed in Scotland. It is conveyed by ticks (*Ixodes persulcatus*). The autumnal encephalitis in Russia is identical with the Japanese Type B.

Mumps. The virus of mumps appears to be a common cause of encephalitis, particularly of the milder sporadic cases, under certain conditions. Kane and Enders (1945), using a complement-fixation test, studied the antibody content of the serum in 51 miscellaneous cases showing clinical manifestations of meningoencephalitis. Positive reactions were obtained in all of the 17 patients who showed salivary-gland enlargement, and also in 16 patients without evident involvement of these glands. Negative reactions were obtained in the remaining 18 cases, and some agent other than mumps was presumably involved. A previous subclinical and unrelated attack of mumps is not entirely excluded, but is not likely to have occurred in all these cases.

Laboratory Diagnosis of Encephalitis. A definite diagnosis of the type of encephalitis can be made only by isolation of the virus from the brain after death or by demonstrating protective antibodies in the serum during convalescence. In the severer cases the blood usually shows a polymorphonuclear leukocytosis. The spinal fluid is usually under increased tension, may be hazy, and shows an increase in globulin and in cell count (50 to 2000). In the early stages of infection there are often many polymorphonuclear cells but later mononuclears predominate. In the Economo type the changes are often slight, and the fluid is usually normal in the chronic cases.

Postvaccinal Encephalitis. Since 1922 many cases of encephalitis following vaccination, similar to the types of encephalitis which occasionally follow measles, smallpox, possibly other virus diseases, and antirabic immunization, have been reported. The greatest outbreaks have been reported in England and Holland, where infantile vaccination is not general. Most of the cases occur in children and young adults who have been vaccinated for the first time. Infants seem to be quite immune. In England the incidence has been estimated at less than one case in 33,000 vaccinations. The mortality is slightly more than 50 per cent.

Symptoms begin 7 to 14 days after vaccination with headache and vomiting. Within a week fever, rigidity of limbs, paralyses (first flaccid, later spastic), and coma may follow. Recovery, even after paralyses have occurred, is usually complete in postvaccinal encephalitis.

Scattered areas of demyelinization in the neighborhood of the blood vessels, found particularly in the cerebrum, midbrain, and pons, also in the anterior horns of the cord, occur in all of these types of encephalitis and distinguish them from poliomyelitis and epidemic encephalitis.

There are three theories as to the cause of postvaccinal encephalitis: (1) that it is a manifestation of vaccinia, (2) that vaccination has activated some latent virus present in the body (although such a virus has never been isolated from a case), and (3) that an extraneous neurotropic virus has contaminated the vaccine lymph. Most authorities favor the second theory. Points against the first view are: (1) that the same type of encephalitis occurs after spontaneous infection with other virus diseases, and (2) that its appearance as a postvaccinal complication is recent, although vaccination has been used for more than a century. By passing vaccinia virus through a series of animals by intracerebral inoculation, a neurotropic strain can be produced. The histologic changes which it pro-

duces, however, are said to be different from those found in postvaccinal encephalitis. Against the third theory is the fact that no particular strain of lymph can be implicated.

Benign Lymphocytic Choriomeningitis. In 1925 Wallgren reported under the term "acute aseptic meningitis" a clinical syndrome characterized by an acute onset with fever, severe headache, stiffness of the neck, and vomiting. Blurring of the optic discs, a positive Kernig's sign, and reflex disturbances sometimes occurred, but no paralyses. The cerebrospinal fluid showed increased pressure, was clear or slightly hazy, and showed a slight increase in globulin and a lymphocytosis of from 60 to 2000 (usually about 250) cells. Recovery almost invariably occurred, usually by lysis, after one to three (up to seven) weeks.

In 1935 Rivers and Scott isolated a virus from the spinal fluid in two such cases, which was found to be identical with a virus obtained by Armstrong and Lillie in 1934 from a monkey, and by Traub (1935) from a colony of white mice. The same virus has since been repeatedly isolated, but it has been demonstrated as the etiologic agent in less than a third of the cases presenting this clinical picture. It has since been shown by inoculation of human volunteers and by clinical studies that the virus causes a systemic infection in man, characterized by fever, prostration, and grippe-like symptoms—occasionally those of an atypical pneumonia. The virus is then present in the blood. In many, probably most cases recovery occurs after one to three weeks. In a minority of the cases, sometimes after a short remission, symptoms of nervous-system involvement appear. In rare instances manifestations of encephalitis have developed, with permanent sequelae. Autopsy has been performed on a few subjects who died following illness characterized by these symptoms, but the identity of the virus was not positively established.

Recovery from both types of infection is followed after two weeks or more by the appearance of neutralizing antibodies in the serum, and this has been used to identify atypical mild cases. Such studies indicate that mild cases are relatively common. Armstrong (1939–1941) reported finding antibodies in 10 per cent of 1000 persons who gave no history of disease of the central nervous system, and in 26 per cent of 106 persons with a history of recent infection of the upper respiratory tract.

Epidemiology. The usual mode of transmission in man is not known. The virus causes epidemics in mice under natural conditions. Traub showed that in a colony of white mice the virus was transmitted to the young *in utero*. The young mice often showed signs of illness from which they usually recovered, but adult mice rarely showed any symptoms. The virus persisted in the tissues, however, and was excreted in the nasal secretions, urine, and feces throughout life. The same is true of gray house mice, and infected mice have been trapped repeatedly in the homes of patients having the disease. Neutralizing antibodies have been observed to develop in the serum of caretakers of infected mice. It is probable that the virus can be passed back and forth between the species, and it seems likely that the mouse constitutes the natural reservoir of the virus, since the latter is better adapted to mice than to man. No direct proof of this is available, however.

The virus passes through Seitz pads and Berkefeld *V*, *N*, and *W* filters. Its diameter is estimated at 70 to 80mμ or less. It resists freezing and desiccation and retains its infectivity in 50 per cent glycerin for long periods. Mice can be readily infected by intracerebral inoculation, showing signs of illness after six to seven days and usually dying with convulsions a day or two later. Guinea pigs can readily be infected by subcutaneous or intraperitoneal as well as by intracerebral inoculation. Symptoms appear after eight days; in some there is merely a febrile reaction, in others severe illness with dyspnea, salivation, conjunctivitis, somnolence, and death. The meninges and choroid plexus are densely infiltrated with lymphocytes, but the cortex shows little change. Frequently there are areas of interstitial pneumonia, pleural effusions, focal necroses in the liver, and some-

times myocardial lesions. Animals which survive are immune. Mice can be immunized by subcutaneous injections, but usually show only trivial signs of illness.

Laboratory Diagnosis. This depends upon the demonstration of the characteristic changes in the cerebrospinal fluid, the exclusion of bacterial types of meningitis by cultures and acid-fast stains of the sediment, and the production of the disease in animals by injections of spinal fluid either intracerebrally into several mice or (preferably) subcutaneously into guinea pigs.

Poliomyelitis. This disease is caused by a filtrable virus which in a large majority of the cases appears to be limited mainly to the gastrointestinal tract. Symptoms are usually mild and not distinctive, and these "abortive" cases are rarely recognized except in epidemics in which severe attacks of the disease occur. In a relatively small minority of the cases the virus invades the nervous system, and the familiar severe form of the disease develops.

Clinical Picture. The incubation period of the severe classical form of the disease ranges from 3 to 14 (usually 7) days. The onset is usually abrupt, with fever, malaise, headache, and often gastrointestinal disturbances. During this prodromal period there may be signs of meningeal irritation, such as muscular twitching or tremor, and stiffness of the neck and spine, but the symptoms are not distinctive. After two to three days, the characteristic flaccid paralyses appear and quite rapidly reach a maximum, from which as a rule an incomplete but often substantial degree of recovery slowly occurs. Every possible gradation in severity is seen. Death from respiratory failure may ensue if the respiratory muscles are extensively involved.

Pathology. The pathologic lesions in the nervous system consist of localized areas of ganglion-cell destruction with perivascular and interstitial small-cell infiltration of the leptomeninges and gray matter of the cord. Ledingham (1935) believes that this infiltration is a secondary response to injury of the nerve cells by the virus. The lesions involve primarily the motor cells in the cortex, midbrain, medulla, and anterior horns of the spinal cord. The virus has been repeatedly isolated from the tissues of these regions. Sabin and Ward (1941) reported finding the virus also in the contents of the descending colon (regularly) and often in the pharyngeal mucosa and the washed wall of the ileum, and (once) in the washed wall of the descending colon. They found it rarely or not at all in many other tissues examined, including the nasal mucosa.

Pathogenicity for Animals. A typical poliomyelitis can be produced in monkeys by inoculation of filtrates of infected tissues from human beings, either by the intracranial, intravenous, intraperitoneal, subcutaneous, or most readily by the intranasal route. The virus can be maintained indefinitely by serial inoculations. In the monkey experimentally infected, the virus is widely disseminated in the early stages of the disease, and has been demonstrated in the blood, cerebrospinal fluid, lymph nodes, and nasopharynx. In man, however, it has not been demonstrated in the blood or spinal fluid. Other laboratory animals cannot be infected by material from human cases. In a very few instances, however, such as in the Lansing strain, the virus after isolation in monkeys has been adapted to mice and then maintained in them by serial intracerebral inoculations. Such adapted viruses, which are probably mutants, differ from the original strains in various respects—for example, in having a lower virulence for monkeys.

Epidemiology. Until recently it was believed that the virus was disseminated by droplet infection by persons having abortive attacks and by carriers. This was based largely on observations in monkeys following intranasal inoculation. In the monkey the virus probably penetrates the olfactory cells and passes to the brain centripetally through the nerve fibers, which were supposed to serve also as pathways for the centrifugal passage of the virus to the exterior (nasal mucosa). There is now strong evidence that this occurs rarely if at all in man, and that the gastrointestinal tract constitutes the portal of entry and primary locus of infection. It is still disputed whether this locus is the pharyngeal mucosa, the wall of the small intestine, or both. The virus is present in high concentration in the feces, and survives for long periods in sewage. It has been repeatedly demonstrated in the sewage of large cities during epidemic periods. Flies may serve as mechanical carriers of the virus. Actual infection of human beings from these extraneous sources has not been demonstrated, although it seems quite possible. There is strong epidemiologic evidence, however, that in recent epidemics, in which young children were predominantly affected, the virus was spread among them by fecal contamination. Contaminated saliva or pharyngeal secretion, spread by direct contact or by droplet infection, is probably also important. The virus has been repeatedly demonstrated in the feces and pharyngeal secretion of healthy contacts.

Mice are subject to a natural infection closely resembling human poliomyelitis. This infection is caused by a virus (Theiler's virus) intimately related to that causing the human disease. This virus is likewise present in the gastrointestinal tract and feces. It has been suggested that the human poliomyelitis virus and Theiler's virus have a common origin, and that the human disease originally may have been acquired from mice. There is no evidence, however, that at present the viruses can be passed back and forth between the species, or that mice or any other animals serve as a reservoir for the usual strains of human poliomyelitis virus. Jungeblut and Dalldorf (1943), however, in a small epidemic, obtained apparently identical filtrable viruses from the brain of one patient and from gray mice captured on the premises on which the patient resided. Both viruses were readily adapted to white mice in which they caused typical poliomyelitis. Both were neutralized by sera from three convalescent patients, and in lesser degree by antisera to Theiler's mouse-encephalitis virus and to murine and simian strains of poliomyelitis virus originally derived from persons suffering from poliomyelitis. At present, this must be regarded as probably an exceptional occurrence.

The factors which determine invasion of the nervous system are not well understood. Possibly the development (by mutation) of an unusually virulent or invasive strain may be the determining factor in some cases. The virus probably reaches the brain and spinal cord by centripetal passage through nerve fibers. In a number of cases tonsillectomy has been followed promptly by a severe attack of the disease, and it seems probable that minor abrasions or other injuries elsewhere in the gastrointestinal tract might expose nerve fibers to invasion by the virus.

The virus is one of the smallest known, estimated at from 8 to 12mμ in diameter. There is some evidence that it has a slender rod shape. It is readily destroyed by heating to 50° or 60° C. for 20 to 30 minutes. It resists desiccation

and lives for long periods in water, milk, or sewage. It remains viable for years in tissue preserved in 50 per cent glycerin and kept at 4° C. Different strains vary in their virulence for animals and, according to Flexner, this may fluctuate inexplicably over a period of years. Antigenic differences between different strains have been described.

Immunity. An attack of poliomyelitis confers a lasting immunity, and the serum of a human being or a monkey after recovery contains virus-neutralizing antibodies. A survey of adult urban populations has shown neutralizing antibodies in a large proportion (about 75 per cent) of individuals having no history of the disease. This is believed due to a previous subclinical or abortive attack, and probably to repeated exposures to the virus.

Laboratory Diagnosis: cerebrospinal fluid. In the prodromal period of the disease there may be no abnormality in the fluid obtained by lumbar puncture. Occasionally the pressure of the spinal fluid is increased. With the advent of meningeal involvement, however, the character of the fluid changes. It is usually clear, or there may be a faint haziness by transmitted light. The cell count is increased, varying from 20 to 1000, or even 2000 cells per cu. mm. At first these cells may be predominantly polymorphonuclears, but after 24 or 36 hours these are replaced by lymphocytes. The globulin is increased; glucose and chlorides are normal. A delicate clot may form in the fluid. Stained films show no bacteria. The virus has not been demonstrated in spinal fluid by animal inoculation.

blood. During the early phase of the disease there is usually a moderate polymorphonuclear leukocytosis (15 to 30,000) with a reduction in the relative number of lymphocytes. The virus has not been found in the blood in human cases, although in the experimental disease in monkeys it has been demonstrated in very early cases.

Serum Treatment. Serum treatment has been tried extensively, both by intravenous and intrathecal injection, using serum from convalescents and pooled normal adult serum. The results have been conflicting and unconvincing. Intrathecal injections have given no better results than intravenous, and some believe them actually harmful. All agree that serum is useless after paralyses have developed. Diagnosis is open to question in the preparalytic stage, and it is impossible to predict which patients will develop paralysis without treatment. Evaluation of the results is therefore difficult. In monkeys serum has no curative value, even when given very shortly after inoculation of the virus, and the same is probably true in man. Serum may have some transient prophylactic value, but this is also difficult to demonstrate.

Vaccination. Since Flexner, Amoss, and Rhoads demonstrated that animals could be effectively immunized by the injection of an attenuated virus, and by sublethal doses or by multiple intradermal injections of an active virus, vaccination of human beings has been attempted. Those viruses which have been completely inactivated have shown little or no antigenic power in monkeys and presumably would be of no value in man. Using vaccines which have been attenuated in various ways, but are still viable, the production of virus-neutralizing antibodies has been stimulated in animals and man, presumably with the development of immunity. Attacks of poliomyelitis have followed the use of such vaccines, however, and the procedure has been regarded as too dangerous for general use. Using a virus completely inactivated by ultraviolet radiation, Milzer et al. (1944) reported the successful immunization of mice, with the development of neutraliz-

ing antibodies in their serum (Lansing strain). The value of vaccines in man has not yet been demonstrated.

Rabies. Rabies is primarily a disease of dogs, wolves, cats, and other carnivora, but is communicable to man and domestic animals by the salivary secretions of rabid animals. It is conveyed most commonly by bites, but infections have occurred from the licking of apparently normal skin by rabid dogs. Epidemiologically dogs are almost exclusively the source of infection.

In man the period of incubation is stated to be from two weeks to six months, but is usually less than six weeks. The first symptoms are irritability and depression, with early difficulty in deglutition. These are succeeded by extreme restlessness and hyperesthesia. The dread of drinking water (hydrophobia) is due to painful reflex spasms. The temperature ranges from 100° to 102° F. The stage of excitement lasts about two or three days and is followed by a paralytic stage which lasts a few hours and ends invariably in death.

An unusual paralytic type of rabies in man and cattle has been reported in Trinidad and also in South America. It was shown (Lima, 1934) to be transmitted by the bite of the vampire bat, *Desmodus rotundus*.

In the dog the first symptoms are a change in disposition, followed by excitability and often combativeness, and ending almost invariably in paralysis and death within 10 days, and often within five days.

Virus of Rabies. Rabies is caused by a neurotropic virus which is filtrable with some difficulty through Berkefeld *V* filters. It can be preserved for months in 50 per cent glycerin. If frozen and desiccated rapidly it maintains its virulence, but if dried at room temperature it loses its virulence within a week. Marked differences in virulence occur in various strains of street virus, and may be produced by adapting it to different species of hosts, but all types are antigenically related.

After inoculation the virus apparently travels slowly to the central nervous system by way of the axis cylinders of the peripheral nerves. Inclusion bodies are practically always demonstrable in the cytoplasm of the cerebral cells. In the dog the virus is constantly present in the saliva, which may be infective five days before symptoms appear. It is believed to reach the salivary glands by way of the nerves. Virus has very rarely been demonstrated in the blood. Remlinger and Bailey (1939) reported demonstrating the virus in two ticks (*Ripicephalus sanguineus*) which had been allowed to bite a rabid dog. In man the virus has been demonstrated in the saliva by several observers but not by others. It has been demonstrated in the salivary glands.

Diagnosis. The symptoms and death of a rabid dog are important points in diagnosis; therefore, the suspected animal should be kept under observation. If it is killed prematurely, the characteristic histologic changes in the brain may not have developed sufficiently to permit a diagnosis to be made. After the animal dies, the head and several inches of the neck should be removed and sent to the nearest laboratory. The head may be packed in ice, or the brain may be removed, sectioned, and placed in a mixture of equal parts of glycerin and water which has been sterilized by boiling and allowed to cool. This will preserve the virus for months.

When examining or handling rabid material all possible precautions must be taken; goggles and heavy gloves should be worn, and all contaminated material should be incinerated. To open the skull, the skin and muscles are dissected from the top down to the ears. Then a transverse cut just back of the eyes is made with a cleaver; other cuts are made on either end of this, carrying these lateral incisions back to the ears. The flap so formed is removed with bone forceps. The entire brain is removed from the skull and one hemisphere is incised longitudinally so that a lateral ventricle is opened. In the floor of this will be found the hippocampus which appears as an elevated mass. This structure is cut through transversely; it will seem to consist of concentric dark and light rings. A small piece of a dark ring is removed and placed on a clean slide. It is mashed with a second slide and a smear is made by dragging the top slide across the length of the bottom slide. Impression slides can also be made by pressing a clean slide firmly against the transverse area.

The slides are stained immediately (while still moist) in a mixture made as follows:

Basic fuchsin (saturated solution in absolute methyl alcohol)	2–4 ml.
Methylene blue (saturated solution in absolute methyl alcohol)	15 ml.
Methyl alcohol (absolute)	25 ml.

The methylene blue and the methyl alcohol are first mixed and then 2 ml. of the fuchsin are added and a test stain is made. Ideally the thin portions of the smear should be violet in color rather than blue; 0.5-ml. amounts of the remaining fuchsin solution are added until this effect is attained. The slides are dipped in the stain for three to four seconds only and rinsed in tap water for another second, then dried without blotting.

The Negri bodies, typical of rabies, will appear as bright-red inclusions within the blue nerve cells. They may also be demonstrated with Giemsa's stain. Occasionally they will not be present in the hippocampus but can be found in other areas so that it is wise to examine smears from the cerebral cortex and from the cerebellum before rendering a negative report.

If the Negri bodies cannot be found because of putrefaction or other reasons, the Gasserian ganglia should be examined. In typical lesions the ganglion cells are more or less completely destroyed and replaced by cells of other types.

For animal inoculations white mice should be used. A suspension of the hippocampal tissue in saline is made and a series of mice are injected intracerebrally. About 0.03 ml. per mouse is used. Intracerebral injections are made by lightly anesthetizing the mouse and forcing a tuberculin-size needle just through the skull a little anterior to the ears and slightly to the side of the midline. Mice die from rabies usually in from 6 to 10 days but survivors should not be discarded before the thirtieth day. The brains of those dying should be examined for Negri bodies as described above. It is wise to inoculate a number of mice (three to five) with each specimen because the mortality from trauma after intracerebral inoculations may be quite high.

NEGRI BODIES. Diagnosis depends primarily upon the demonstration of these inclusion bodies in the cytoplasm of the brain cells. They are practically constantly present and are characteristic of rabies. They are round or oval in shape, from 1 to 20μ (usually 4 to 10μ) in diameter. They have a homogeneous bluish-staining background containing pink corpuscles which include minute reddish rods and granules. They may be found anywhere in the brain, but are most numerous in the following areas, in order: (1) cornu Ammonis (hippocampus major), (2) region of the fissure of Rolando (in the dog, the crucial sulcus), and (3) the cerebellum. In street rabies, large forms (20μ) may be found, whereas in animals inoculated

with "fixed" virus, only minute forms (0.5μ or less) may be detected. Negri bodies have been found four to seven days before the onset of symptoms.

Since the relation of the bodies to the nerve cells is more or less disturbed in making smears, examination of stained sections is preferable. A bit of brain tissue is fixed for five to seven hours in Zenker's fluid, washed and dehydrated in graded alcohols and chloroform as usual, embedded in paraffin, and sections cut. These may be stained with Giemsa's stain. It is necessary to differentiate in 95 per cent alcohol. The Negri bodies are brought out as lilac-red bodies in the blue cytoplasm of the nerve cells.

Local Treatment. Thorough cauterization of the dog-bite wound with pure nitric acid (no other cautery is efficient) must be carried out as soon as possible after the bite, even if Pasteur treatment can be given later. Immunization should be started immediately after the bite has occurred, although the mortality has not been definitely higher in individuals whose immunization was somewhat delayed.

Pasteur Treatment. By subdural inoculation of rabbits in series the virulence of the virus for rabbits is finally so increased that the rabbits die in six days. It is impossible to increase further the virulence of the virus, which is then termed "fixed virus." The pathogenic power of this virus for other animals is also changed so that it is not likely to cause rabies if injected subcutaneously.

To attenuate this virus, the spinal cord of the rabbit is removed and dried over caustic potash at a temperature of 23° C. The cord is divided into segments about 1 inch in length. Drying for about 15 days seems entirely to destroy the virus. To prepare the material for prophylactic injections a small portion of the cord is emulsified with normal salt solution. By the usual American method the first subcutaneous injection is of a cord which has been desiccated for eight days. The treatment is given daily for about 20 days. The immunity is "active," and the immunizing agent is a "vaccine." The activity of the virus can be preserved for about a month by glycerin and cold storage. This method is now rarely used in the United States.

Other methods of treatment are:

1. Harris' Method. In this the brain and cord are frozen by means of carbon-dioxide snow, ground up, and dried over sulfuric acid for about two days. The virulence of the virus is reduced one-half. The virus, if kept at 0° C., retains the same potency for at least six months.

2. Cumming's Method. In this the brain is emulsified in saline and dialyzed with formaldehyde solution. The virus is so attenuated that intracranial inoculation does not produce rabies.

3. Högyes' Method. The fresh virulent cord is injected but so diluted in strength that it acts as an attenuated virus.

4. Phenolized Virus. Fermi, and more recently Semple, have used virus which has been inactivated by the application of strong (1 or 2 per cent) phenol. Before injecting, the carbolized emulsion is diluted to preservative strength (0.5 per cent). This method is now used extensively for man and for prophylactic immunization of dogs.

Results of Treatment. Only a minority of persons who are bitten by a rabid dog and are not treated develop rabies although the actual incidence is not known. Cornwall reported a series of 423 persons (in India) bitten by dogs known to be rabid, of whom 148 developed rabies. Others, however, believe the usual incidence to be much less than this. Lacerated bites about the face, neck, and upper extremities are the most serious.

The mortality from rabies in cases of dog bite which receive proper prophylactic treatment is less than 1 per cent. It is practically impossible, however, to secure an adequate series of untreated cases as a control, and the degree by which the mortality is lowered is not accurately known. This is generally believed to be substantial, but there are still some skeptics (Webster, 1942). Andrewes (1944) states, "It would seem advisable to keep an open mind in this matter."

Thus far no one of these methods of immunization has been proved to be superior to the others. Neutralizing antibodies have been demonstrated in the serum of individuals after immunization, but not in victims of the disease.

A *postvaccinal encephalitis,* similar to that which occasionally follows vaccinia, may occur but is rare. McCoy found records of only three cases in a series of 20,000 persons treated in this country. It has been suggested that this encephalitis is due to an allergic reaction to the brain tissue in the vaccine. See p. 294.

Influenza. The term "influenza" commonly has been used to indicate a clinical syndrome rather than an etiologic entity. In typical form it is a highly contagious disease coming on abruptly with fever, malaise, prostration, generalized aches and pains in the back and muscles including the eye muscles, and signs of irritation of the respiratory mucous membranes which may be slight in comparison with the systemic symptoms. There is usually a leukopenia and a marked susceptibility to secondary bacterial infections, particularly with streptococci, pneumococci, and Pfeiffer's bacilli, which are almost invariably the immediate cause of death in fatal cases.

Illness of this type may appear in great pandemics such as occurred in 1889 and 1918, in relatively localized epidemics (commonly recurring at intervals of two to five years) which may have a high morbidity in the areas involved, and as sporadic or endemic cases. Positive recognition of the latter is difficult because of the lack of any distinctive physical signs. Epidemiologic evidence suggests that during interepidemic periods the virus is maintained in carriers, but this has not been proved.

Etiology. The cause of pandemic influenza has not been demonstrated. There are many excellent reasons for believing that the disease is due to a virus, but this is still only an inference.

By using a susceptible animal, the ferret, Wilson, Smith, Andrewes, and Laidlaw (1933) were able to reproduce the epidemic type of the disease by intranasal inoculation of filtered nasal secretions from influenza patients. They transmitted the infection serially in the ferrets by intranasal inoculation and by contact, but not by any other route. The virus attacks the superficial epithelium of the respiratory tract in man and animals, spreading "like a prairie fire," but it does not invade the blood stream or the organs generally. In animals, virulent strains may cause focal areas of pneumonitis, but in man, in most cases at least, pneumonia is believed due to secondary invaders. After recovery the animals are immune to reinfection and their serum contains neutralizing antibodies.

In 1934 Andrewes et al. produced infection in mice by intranasal inoculation of virus after it had been established in ferrets. Anesthetization increased their susceptibility and

predisposed to the development of pulmonary lesions. This virus, now known as influenza A, has since been isolated many times from epidemics in this country and elsewhere.

In 1940 Magill and Francis independently isolated from epidemic cases another virus, influenza B, which is antigenically distinct from A. Although virus B has been reported less frequently than A, it is widely distributed in this country and has been the cause of a number of epidemics. It has also been reported in England and Australia.

Subsequent studies indicate that lesser antigenic differences, which may be well marked, exist between various strains of A virus, and the latter should perhaps be regarded as a group of related viruses rather than a single organism.

There remains a substantial number, about a third, of epidemic cases from which neither A nor B virus can be isolated, and their etiology is not known. The same is true of most of the sporadic cases.

Virus of Influenza. The virus passes through the usual filters. Its diameter has been estimated at about 120mμ. More recent studies, however, suggest that it is probably only one-tenth of this size. Chick embryos are highly susceptible to infection, and Burnet has utilized injections of filtered secretions into the amniotic cavity as a means of isolating the virus from human cases. After the virus has been adapted to the chick embryo, it will also grow readily in the allantoic fluid, which serves as an abundant source of virus in relatively pure form. Hirst (1941) showed that active virus causes agglutination of chicken red-blood-cell suspensions, and also that this agglutination is specifically inhibited by immune serum from man or animals. This procedure has been used extensively for titrating the antibody content of sera and has proved to be a valuable substitute for mouse-protection tests.

Influenza A virus is a highly labile and variable organism. Burnet in particular has stressed the changes in the virus which occur after its isolation. In the original (O) form, as obtained from man, it is nonpathogenic for mice; it grows well in the amniotic but not in the allantoic cavity of the chick embryo; and it agglutinates human and guinea-pig red cells more strongly than chicken cells. In the altered (D) form these characteristics are reversed. It is probable that antigenic changes in the virus also occur in the process, and may explain in part the strain differences which have been observed. This change Burnet attributes to a mutation and not to a process of gradual adaptation, the new mutant form outgrowing the O form under the artificial conditions in the laboratory.

With continued transfer of the virus there appears also to be a gradual loss of virulence for man. This may be an advantage from the standpoint of human immunization if an attenuated living virus is to be used to produce a subclinical infection. It may be a distinct disadvantage, however, if inactivated virus is used, since the reduced virulence and antigenic changes may impair the effectiveness of the vaccine, as is well known to be the case, e.g., with typhoid and pertussis vaccines.

Immunity. There appears to be relatively little natural immunity to influenza, since there is often a high morbidity at the beginning of an epidemic in isolated communities, among members not previously exposed. Recovery from an attack is followed by an immunity to the same type of virus, associated with the appearance of protective antibodies in the serum. This immunity appears to be temporarily effective, but less enduring than that observed after many other virus diseases. In some individuals successive attacks have been due to antigenically different types of virus. Several instances have been reported, however, in which A virus

was isolated in two and even three successive attacks in the same individual at about two-year intervals. In ordinary communities, however, a substantial immunity is gradually built up, since even in severe epidemics only a minority of individuals are attacked.

Vaccination. In spite of a great deal of effort, attempts to immunize human beings by vaccination have been only partly successful. Recent attempts have been largely concentrated on the preparation of killed vaccine which can be administered subcutaneously. Technical difficulties have been encountered in obtaining adequate concentrations of virus and in finding procedures which will inactivate it without weakening its antigenic activity excessively. Sawyer (1945) reported that by centrifugalizing allantoic fluid of infected chick embryos at high speed, high concentrations of virus can be obtained. He found that ultraviolet radiation or the addition of 1 part in 2000 of formalin and 1 in 100,000 of phenyl mercuric nitrate were the most satisfactory methods of inactivation for large-scale production.

During the epidemic of 1943–1944, under the auspices of the United States Army Commission on Influenza, a large number of volunteers were inoculated subcutaneously with a concentrated inactivated vaccine containing A and B viruses. Most of the vaccine administered under the U. S. Army Commission was a formolized product cultivated in chick embryos and concentrated by adsorption on the red cells of the embryo and subsequent elution in one-tenth the original volume of fluid. The incidence of clinical influenza in the vaccinated individuals was 2.22 per cent as compared with 7.11 per cent in the controls. This protection appeared to be fully effective, however, for only a brief period. In the group contracting influenza two weeks after vaccination, the incidence was reduced by 85 per cent as compared with the controls. After six weeks the incidence was reduced by only 40 per cent, and after a year by 35 per cent. The incidence of influenza (even a year after vaccination) was significantly lower, however (1.9 per cent as compared with 12.4 per cent), in groups half of whose members had been vaccinated than in other similar groups comprised only of unvaccinated individuals. It may be that the reduction of infection among the half of the group who were vaccinated indirectly lowered the incidence of infection among the unvaccinated half of the same group by reducing the frequency of exposure and thus tended to conceal the full effectiveness of the vaccination (Francis, 1946). Although the titer of protective antibody had fallen below the maximum, occurring two weeks after vaccination, yet after a year the vaccinated individuals still had antibodies "well above the prevaccination level."

At present, vaccination with inactivated vaccine is most useful just before or at the onset of an epidemic. Improvements in technic will probably increase to some extent the degree and duration of protection. A serious obstacle to effectiveness, however, is the fact that the protection presumably is due to circulating antibody, and influenza virus infects without coming into contact with the blood. The only antibody that might be expected to furnish protection is that found in the superficial epithelial cells or in the fluid that covers them. Some antibody is secreted into this fluid, but it is in much lower concentration than in the blood. There is as yet no proof that a subclinical infection produced by a living virus sufficiently attenuated to be reasonably safe yields any better immunity than the killed vaccine.

Swine Influenza. In 1931 Shope isolated a virus from swine affected with influenza, which is immunologically related to but different from influenza A virus. Both mice and ferrets are susceptible. In swine influenza a Gram-negative bacillus, *Hemophilus suis,* is associated with the virus. Shope showed that the typical severe disease in swine depends upon the combined activity of the virus and the bacterium. The virus alone causes only an ill-defined mild transient infection that is often afebrile, and the bacillus alone produces no ill effects. Swine influenza was first recognized in the United States at the be-

ginning of the pandemic of 1918, and Shope suggested that the virus was transmitted from man to swine at that time. No conclusive proof of this has been obtained.

Shope also showed that the swine influenza virus enters the lung worm of the swine and is transmitted in the ova to the earthworm, the intermediate host of the parasite. When the infected earthworm is eaten by a pig, the virus is carried to the lungs and liberated. Here it may remain latent ("masked") until some nonspecific stimulus, such as cold stormy weather, starts up its multiplication and the development of clinical disease.

Common Cold. The weight of evidence at present strongly favors the view that colds are due to a filtrable virus.

In 1914, Kruse, and later Foster, Dochez, and others, demonstrated that colds could be produced in man and monkeys by intranasal inoculation of the filtered nasal discharge from persons with acute coryza. Dochez, and others, found that the virus multiplied in chick-embryo medium, and after many transfers they were again able to reproduce the disease by inoculation.

It may be assumed that human beings possess some natural immunity to the virus, and that infection takes place more easily when this is lowered by fatigue, exposure, etc.

Measles. This disease is believed to be caused by a filtrable virus, since typical measles has been transmitted to human volunteers by filtrates of the nasopharyngeal secretions, and of the blood at the height of the disease. An atypical form of the disease has been produced in monkeys and transmitted serially. After recovery they have been shown to be immune. The ordinary laboratory animals are not susceptible, but infection has been reported in rabbits after intratesticular inoculation.

The serum of convalescents contains protective substances which are effective in preventing the disease when given within a few days (three to five) after exposure. If given later the attack is modified and mild, yet produces an effective immunity. The serum is given in a dose of 2 to 15 ml. (2 ml. per year of the child's age). Since almost all adults have had measles their blood may be used when convalescent serum is not available. Adult serum, however, is somewhat less effective and double quantities are recommended. The globulins in the normal human placenta have similar protective power, and a preparation of this has been marketed.

Epidemic Parotitis (Mumps). Wollstein demonstrated a filtrable virus in the saliva of patients during the first three days of the disease (occasionally up to the sixth day) with which she produced suggestive lesions in the parotids and testicles of cats. Injection of the filtrate into the salivary ducts of monkeys caused a disease resembling mumps which could be transmitted serially. These observations have been amply confirmed. The virus was neutralized by the sera of persons convalescing from mumps, and complement-fixation reactions may be obtained with such sera. The virus occasionally causes an encephalitis (see p. 201).

Primary Atypical Pneumonia. This term is commonly applied to a group of respiratory infections which differ clinically from those of pneumococcal or other recognized bacterial origin. There is usually a more gradual onset with increasing fever, malaise, often severe headache, and a nonproductive cough, but without a chill, pleural pain, herpes, or bloody or rusty sputum. In severer cases there may be marked prostration, dyspnea, rapid respiration, cyanosis, and severe cough with tenacious mucoid sputum. Physical examination usually shows only slight abnormalities, but roentgenograms of the chest reveal relatively more marked

and widespread changes consisting of thickened bronchial markings, shadows in the hilus region, or scattered patchy areas of consolidation in the more peripheral parts of the lung fields.

The leukocyte count usually gives normal values. Blood cultures are negative, and in uncomplicated cases no known pathogenic organism of significance can be demonstrated in the sputum. In about half the cases during convalescence "cold" autohemagglutinins can be demonstrated in significant titer in the serum (see p. 411).

The infection is not influenced by sulfonamides or penicillin, but the mortality is low. The disease usually terminates by lysis after about 10 days, but may persist for three or four weeks, occasionally much longer, and the disability may be out of proportion to the severity of the acute symptoms.

There is increasing epidemiologic evidence that pneumonia is only an occasional manifestation of this infection, and that the infection is often limited to the upper respiratory passages or bronchi. In such cases, clinically it cannot be differentiated with any certainty from ordinary influenza or the common cold.

Etiology. In a majority of the cases the etiologic factor is still uncertain. In a small number (less than 5 per cent) viruses related antigenically to the psittacosis virus have been demonstrated. Even more rarely cases have been caused by the virus of lymphocytic choriomeningitis and by the rickettsia of Q fever. Several investigators have reported the isolation of other viruses in limited groups of cases by the intranasal inoculation of the mongoose, cotton rat, hamster, or young guinea pigs. In these experiments serious technical difficulties have been encountered in that the pathogenicity of these "viruses" for all the ordinary laboratory animals is very slight, and serial passages were usually required to obtain them. Under such circumstances there is great danger of confusing or contaminating the virus with other respiratory viruses to which these animals are naturally subject, and these early reports are not regarded as convincing.

Eaton et al. (1945) have recently reported the isolation of an agent by serial inoculation of chick embryos with filtered sputum or human lung suspensions. The virus caused no distinctive changes in the chick embryos, and to demonstrate its presence they had to utilize intranasal inoculation of suspensions of the trachea, lungs, and amniotic membranes into hamsters or cotton rats, in which the virus caused the development of small pulmonary consolidations. Similar lesions were produced by inoculation of these animals with infected sputum, but the virus could not be maintained in these animals. Eaton and his co-workers advance evidence that the agent is distinct from the viruses of influenza, the psittacosis group, and the pneumonia-producing viruses naturally infecting these animals. They reported the demonstration of neutralizing antibodies for this virus in the serum of 42 out of 69 patients during convalescence (but not during the acute phase) from clinically diagnosed primary atypical pneumonia, both from the eastern United States and the Pacific Coast.

Even if this work is confirmed, it is probable that other unidentified agents are also capable of producing this clinical picture.

The Commission on Acute Respiratory Diseases of the U. S. Army has reported (1945) passing the infection through two series of human volunteers by intranasal inoculation of filtered as well as unfiltered sputum from patients with primary atypical pneumonia.

Psittacosis Group. In this group are included the diseases due to the viruses of psittacosis, ornithosis, meningopneumonitis (of Francis), feline pneumonitis

(of Baker), mouse pneumonitis, lymphogranuloma venereum, trachoma, and inclusion conjunctivitis. Related viruses have been isolated from a few cases of primary atypical pneumonia in man. The viruses of the psittacosis group have certain characteristics in common which separate them rather sharply from other viruses. They are relatively large, from 150 to 300mμ, they show characteristic tinctorial reactions, and they undergo a cycle of development within the cells (which can be observed with an ordinary microscope) accompanied by the formation of characteristic inclusion bodies. Some of them (the last four listed above) are susceptible to the sulfonamides. Antigenically all of them are closely related, but can be differentiated by suitable serologic procedures (except perhaps the viruses of meningopneumonitis and ornithosis).

Psittacosis. Psittacosis is an epizootic disease of parrots and related birds which may be transmitted to man, and less frequently from one person to another.

The disease in man is characterized by fever, pneumonic consolidation, and paralysis and is often fatal. During an extensive epidemic in 1929 and 1930, Bedson, Krumwiede and others proved that the disease was caused by a filtrable virus. Levinthal first described minute coccoid bodies (psittacosis bodies) 0.2 to 0.25μ in diameter in the cytoplasm of reticuloendothelial cells in the spleen, liver, and exudates. The presence of these bodies has been amply confirmed. The bodies are believed to represent the virus. They can be demonstrated by means of Castaneda's stain, with which these elementary bodies stain bright blue whereas the cytoplasm of the cell is pink. Bedson described a cycle of development of these bodies in tissue cultures of mouse spleen.

The infection can be transmitted to rabbits, guinea pigs, and mice by inoculation of blood or filtered extracts of liver and spleen. Rivers produced the disease in mice by intraperitoneal inoculation of filtered sputum from human cases and suggested this as a means of diagnosis. Following the injection of 2 ml. of filtered sputum on three successive days the mice usually die after 7 to 10 days and at autopsy show focal necroses in the liver and spleen in which the coccoid bodies can usually be demonstrated. The virus is present in the feces and nasal secretion of infected animals, and infection in man is believed to be through the respiratory tract (Rivers). Pneumonia has been produced in monkeys by intratracheal and intranasal inoculations. Extreme care must be used in handling infected animals, by wearing gloves, gowns, and masks, to avoid inhalation of the virus, since serious laboratory infections have been frequent. The virus can be cultivated in the chick embryo.

Recovery from infection in man and animals is followed by some degree of immunity. Agglutinins and complement-fixing antibodies have been demonstrated in the serum, and the latter have been used as an aid in diagnosis. Virus-neutralizing power is often weak or difficult to demonstrate.

The disease occurs in birds imported from South America and is widespread among psittacine birds in Australia (Burnet), and in fulmar petrels in the Faroe Islands. It also occurs extensively in aviaries in which these birds are bred. Meyer and Eddie showed that infection is commonly acquired by young birds in the nests. Those which recover continue for a time to excrete virus in the feces and nasal secretions. In many birds after excretion of the virus ceases, it can still be demonstrated in the enlarged spleen and kidneys, and such birds may suffer exacerbations of the disease, which may be fatal. Excretion of virus may be resumed in breeding females, providing a source of infection for nestlings as well as for such adults as may have escaped infection in the nest.

Ornithosis. The ornithosis virus is closely related but antigenically distinct from that of psittacosis. The disease is widespread among pigeons in the United States, England, and Australia. A number of cases of human infection have been reported.

Trachoma and Inclusion Conjunctivitis (Inclusion Blenorrhea). These diseases are caused by viruses which are closely related and morphologically similar to that of psittacosis. The viruses have not been successfully transmitted to other animals, however, nor will they grow in the developing chick embryo.

Lymphogranuloma Inguinale (Venereum). This sixth venereal disease, as it is sometimes called, or climatic bubo, the usual tropical designation, is due to a filtrable virus which was first isolated by Hellerström and Wassén (1930) by intracerebral inoculation of monkeys with pus aspirated from inguinal buboes. The disease is usually conveyed by sexual contact. It has an incubation period of four to five weeks. In males the initial lesion appears as a small ulcer, often overlooked, usually in the coronal sulcus. From here the virus passes through the lymphatics into the inguinal nodes, where it spreads widely into the deep as well as the superficial nodes. Pockets of necrotic material form, and eventually perforate externally, forming indolent and relatively painless ulcers. In women the infection may cause esthiomène with hypertrophy and ulceration of the labia, or it may (often) pass into the deep pelvic nodes, and cause salpingitis, proctitis, and rectal stricture —the ano-genital syndrome. The etiologic identity of these syndromes was not recognized until Frei (1925) devised his test for cutaneous hypersensitiveness. Since then the virus has been obtained from many human tissues: from the initial ulcer, the inguinal nodes, the labia, the urethra, the cervix, the rectal mucosa, from conjunctival exudates, and from the cerebrospinal fluid in cases associated with meningitis. Infection may be acquired by other routes, such as the skin of the finger, the mouth, and probably the respiratory tract. In several cases of accidental laboratory infection reported by Harrop (1941) the disease ran the course of an acute systemic infection. The disease is not limited to the Tropics, but is fairly common in the United States, at least in the southern states and on the eastern seaboard. It is much more common in Negroes, but members of the white race are not spared.

virus of lymphogranuloma inguinale. The virus is filtrable and according to Findlay is from 125 to 175mμ in diameter. It can be grown in tissue cultures and grows readily on the chorioallantoic membrane and in the yolk sac of the chick embryo. The latter affords the best means of obtaining uncontaminated virus in large quantities. Inclusion bodies have been described in the cells of infected exudates, particularly in monocytes, and by Findlay (1938) in the brain cells of mice, in which he described a developmental cycle. This was confirmed by Rake's observations in chick embryos (1942). About 12 hours after inoculation, "initial" bodies appeared within the cells—isolated structures about 1μ in diameter, near the cell membrane, taking a green color with Rake's differential stain. These increased in number, forming small groups which became segregated in small vesicles in the cell cytoplasm, surrounded by a limiting membrane and embedded in a greenish-staining matrix. After these structures had attained a diameter of 4 to 7μ, minute reddish-staining elementary bodies about 0.4μ in diameter appeared within them. Multiplication might go on until the cell was filled, or the cell might rupture, and the elementary bodies then penetrate into other cells and start another cycle of development.

The virus can be maintained by serial intracerebral inoculation of monkeys or mice; the latter are highly susceptible. Lesions can be produced by other methods of inoculation but less regularly.

LABORATORY DIAGNOSIS. Animals and human beings who are infected develop specific immune reactions to the virus. The Frei test is based upon a cutaneous allergic reaction to the virus. Frei used as antigen pus aspirated from a bubo, which was diluted with saline and inactivated by heating at 60° C. An intracutaneous injection of 0.1 ml. of antigen is made on one forearm, and a control injection of saline in the other. A positive reaction is indicated by the appearance of an erythematous macule or papule, occasionally a vesicle, which is maximal after 24 to 48 hours and persists for 5 to 10 days. Vander Veer advised reading the reactions after four to five days, as nonspecific reactions usually subside after this interval. To be significant, the macule or papule should have a diameter of at least 7 mm., and the control must be negative.

There are many obvious objections to the use of lymphogranulomatous pus, and this has been largely supplanted first by suspensions of infected mouse brain, and more recently by material from yolk sacs of infected chick embryos. Such antigens are usually diluted to such a degree that they give a reaction 7 to 10 mm. in diameter in a known positive case, and to guard against hypersensitiveness to egg yolk, it is desirable to use as a control material similarly prepared from an uninfected embryo.

Although some still question the reliability of the test, most observers believe that if properly performed it has a high degree of specificity. The reaction usually becomes positive from two to four weeks after the buboes appear, although in a few cases sensitivity never develops. An individual who has once given a positive reaction usually continues to do so indefinitely, but a few cases of reversion to a negative reaction after effective treatment have been reported. A significant percentage of patients in venereal disease clinics, without clear clinical evidence of lymphogranuloma inguinale, give positive Frei reactions, positive complement-fixation reactions, or both. It is probable that at least a large proportion of these patients have a latent infection with the virus. It is manifest that no immune reaction proves the nature of a presenting lesion, but merely indicates that at some time the individual has acquired the infection.

The virus of lymphogranuloma inguinale is related antigenically to the other viruses of the psittacosis group, and these all may show in varying degree cross reactions with one another. Thus Rake reported false positive Frei reactions in several cases of atypical pneumonia. Clinically the differentiation of infections with these viruses from lymphogranuloma inguinale would usually be simple.

Neutralizing antibodies appear in the serum of infected animals and human beings. This fact has been utilized by Findlay and others in the demonstration of the nature of unusual lesions. *Complement-fixation reactions* of the serum of infected animals or human beings may also be obtained. Using, as antigen, virus from the yolk sac of infected chick embryos, McKee et al. (1940) obtained positive reactions in a large percentage of clinical cases of lymphogranuloma inguinale. The reaction is sensitive and seems to be highly specific, but its dependability is not so thoroughly established as that of the Frei reaction.

Some have attempted to prove the lymphogranulomatous nature of suspected lesions by preparing "antigens" from them, as for a Frei test, and injecting them into subjects known to give a positive Frei reaction. Pus or lymph node tissue from the lesion is mixed with five times its volume of saline and the mixture heated to 60° C. for two hours. The following day it is reheated to the same temperature for one hour and 0.3 per cent phenol is added as a preservative. After the sterility of this extract has been proved by culture, 0.1 ml. of it is given a known Frei-positive (and Ducrey-negative) patient. A positive reaction with such material (if a negative reaction is obtained in normal control subjects) is regarded as evidence that the lesion was lymphogranulomatous in origin. The dependability of this *inverted Frei test* is not as yet generally accepted.

TREATMENT. The virus of lymphogranuloma inguinale (like some other viruses of the psittacosis group) is to some extent susceptible to the sulfonamides. Their clinical value in the treatment of patients is now generally recognized. Sulfonamides will prevent the death of mice infected by intracerebral inoculation of the virus. The brains of such mice, however, show lesions similar to those in untreated mice, and contain the active virus. The sulfonamides exert a virostatic rather than virocidal effect, and possibly induce a chronic carrier state rather than effect a fundamental cure. That the same may be true in human infection is suggested by the persistence of a positive Frei reaction in many treated patients. There is as yet no assurance that these cases are not infectious or that they may not relapse.

Infectious Hepatitis (Epidemic Hepatitis). This includes at least many of the cases formerly known as catarrhal jaundice. The disease is characterized by an incubation period of about 30 days, during which there may be transient prodromal symptoms and fever. There is then a secondary rise in temperature, with headache, malaise, marked fatigue, anorexia, and digestive discomforts followed quickly by jaundice. There are, however, many mild cases without jaundice, which are often overlooked. The liver becomes enlarged and tender. The jaundice usually reaches a maximum within a week and then gradually subsides, as general clinical improvement becomes evident. The early mortality is low, about 0.2 per cent, but rarely death may occur early from massive necrosis of the liver and acute hepatic insufficiency. In most cases recovery gradually occurs over a period of one to three months, and is substantially complete, although in some patients tests of liver function show impairment for indefinitely long periods. In a substantial minority, according to Barker (1945) about a fifth of those with outspoken illness, the disease runs a protracted course of four months to a year or more. The severity of the symptoms varies, but severe relapses may occur, particularly after physical exertion which is poorly tolerated. Late deaths may occur, but in most cases recovery eventually takes place. The incidence of cirrhosis or permanent serious liver injury is not yet known.

VIRUS OF INFECTIOUS HEPATITIS. The virus is present in the blood before and during the acute illness. It is also present in the feces and (probably less regularly or in lower concentration) in the urine and nasopharyngeal washings. The infection has not been (convincingly) transmitted to any of the laboratory animals, and investigations have been restricted to tests on human volunteers. MacCallum and Miles (1946), however, have apparently established the infection by serial inoculation of rats in which a mild degree of liver injury had been produced by a deficient diet. The agent has not been cultivated. Man can be easily infected by the ingestion of blood or feces containing the agent and less readily by parenteral injections. The agent is filtrable through a Seitz filter and, according to MacCallum and Miles, through a 63mμ Elford Gradocol membrane, indicating that it is a relatively small virus. It is unusually resistant to heat, withstanding inactivation at 56° C. for one hour, and withstands drying and long storage at low temperatures.

EPIDEMIOLOGY. There is strong evidence that under natural conditions the disease is conveyed by ingestion of food or water contaminated with infective feces or urine. Explosive epidemics have occurred, both in military forces and among civilians, particularly in camps or institutions, which almost certainly were conveyed in this manner. Contact infections are fairly common, and droplet infection is possible but not directly proved. There is considerable epidemiologic evidence and some direct experimental evidence

showing that recovery is followed, at least temporarily, by a substantial degree of immunity.

Laboratory Diagnosis. Except in the milder cases the urine is dark and contains bilirubin. The blood serum gives a high icterus index and an immediate positive direct van den Bergh reaction; its bilirubin content is high. Some increase in bilirubin may be found in patients not frankly jaundiced. There is no leukocytosis, but there may be a relative lymphocytosis with the appearance of atypical lymphocytes resembling those occurring in infectious mononucleosis. Tests of liver function usually show impairment. The most useful appear to be the bromsulfalein-retention test, Hangar's cephalin-cholesterol flocculation test, and tests demonstrating an increase of alkaline phosphatase and less regularly of serum globulin. In severe cases the prothrombin time may be increased, and failure to respond to administration of vitamin K is a bad prognostic sign.

Homologous Serum Jaundice (Hepatitis). This form of hepatitis has followed the parenteral injection of blood or blood products from individuals who were clinically normal and had had no recent illness suggesting a hepatitis. Both in its clinical manifestations and pathologic lesions it is practically indistinguishable from infectious hepatitis, except for the prolonged incubation period between the injection and the appearance of severe symptoms and jaundice. This averages about 90 days and may range from six weeks to six months or more. Neefe and Stokes (1945) have described (in inoculated volunteers) mild prodromal symptoms without jaundice appearing two to four weeks after the inoculation. The mortality is low in most epidemics, about 0.2 per cent, although in some it has been much higher.

Virus of Homologous Serum Jaundice. The agent is in the blood before and during the acute illness, and it is readily conveyed to man by parenteral injection, even in minute amounts. Intracutaneous injections of 0.1 ml. of blood have repeatedly conveyed the infection, and even the traces of blood remaining in carelessly cleaned syringes may suffice when these have been used without sterilization for aspiration of blood for routine laboratory tests. The observations of various British workers suggest that this may be the cause of "postarsphenamine" jaundice in patients under treatment for syphilis. It has also been conveyed by feeding blood, but less readily. Findlay and Martin (1943) reported producing the disease by intranasal inoculation of volunteers with nasopharyngeal washings. The infectivity of the feces has not been demonstrated.

The infection has not been transmitted to animals. The agent has not been successfully cultivated. It is filtrable through a Seitz filter, and resists heating at 56° C.

Epidemiology. There is practically no direct evidence as to how this agent (if it is different from that of infectious hepatitis) is conveyed under natural conditions. The theoretical possibility that it might be conveyed by biting arthropods is obvious, but this is not proved. It is also obvious that the virus may be present in the blood of carriers or individuals with subclinical infection. Volunteers who have recently recovered from serum jaundice have been immune to reinfection.

Relation of Homologous Serum Jaundice to Infectious Hepatitis. The great similarity of the agents and the diseases they produce indicates that they are closely related, and has suggested to many that they are identical. This point has not been determined definitely, but the evidence at present indicates that the agents are probably different. This is based upon: (1) the marked difference in the incubation periods; (2) serum jaundice is most readily conveyed by parenteral injection, infectious jaundice by the oral route; (3) contact infections are relatively common with infectious jaundice, but with serum jaundice occur with great rarity if at all; (4) individuals immune from serum jaundice have usually been susceptible to infectious hepatitis and (probably) vice versa. This is based

both upon clinical observations and experimental inoculation of volunteers (Paul et al., 1945; Neefe et al., 1945).

Yellow Fever. Yellow fever is an acute infection caused by a filtrable virus and characterized in the outspoken cases by jaundice due to extensive liver necrosis.

Clinical Picture. In outspoken cases the onset is sudden, after an incubation period of three to six days, with fever, severe headache and backache, malaise, increasing prostration, and an early tachycardia which soon is replaced by a marked relative bradycardia—Faget's sign. There is rapidly increasing albuminuria with oliguria and sometimes anuria. After three to five days jaundice appears, usually moderate in intensity, and the gums and stomach tend to bleed. When bleeding occurs, blood-stained or black coffee-ground material is vomited. There is a leukopenia. The mortality rate in such cases has been reported as from 25 to 75 per cent, death usually occurring during the first week. These cases, however, constitute only a minority of the individuals infected. In most patients the course is either subclinical or marked by a mild grippelike febrile reaction of a few days' duration without any characteristic symptoms. Virus has been isolated from the blood of some of these patients, but the infection can usually be recognized only by demonstrating immune substances in the blood after recovery. In children the disease is usually mild, although severe and even fatal cases may occur.

Recovery, if it occurs, is usually complete, without relapses or sequelae, and is followed in all cases, mild and severe, by an immunity which is high in degree and probably lifelong.

Pathology. The characteristic pathologic lesion is necrosis of the liver parenchyma, usually midzonal, but often very extensive. Acidophilic "inclusion bodies," possibly areas of degeneration, occur in the nuclei of the liver cells, frequently in monkeys and in about 25 per cent of human cases. The liver lesions are characteristic, and in Brazil, routine examination of liver tissue obtained postmortem by means of a viscerotome in patients dying of an acute infection has often revealed yellow fever in localities where it was not suspected.

Virus of Yellow Fever. The filtrability of the agent was first demonstrated by the U. S. Army Commission in Cuba in 1902. Ultrafiltration experiments indicate a diameter of about 22mμ. The virus is quite susceptible to heat and deteriorates rapidly if kept in salt solution at room temperature, less rapidly if 10 per cent serum is added. It cannot be preserved in glycerin. It can be preserved for long periods by desiccating in vacuo and more easily and safely by simply freezing and storing at a temperature below 0° C. It can be grown in suitable tissue cultures, and grows readily in the developing chick embryo.

The virus from African cases of yellow fever appears to be identical with that in America. No strain differences have been demonstrated. There is considerable evidence that Africa was the original habitat of the virus and that it was imported into America in infected mosquitoes brought to this continent with African slaves.

The rhesus monkey is highly susceptible to infection, both through bites of infected mosquitoes and by inoculation. Following either intraperitoneal or intracerebral injections

of natural virus, this monkey develops an infection similar to that in man, with necrosis of the liver, and usually dies within three or four days. Mice are usually not susceptible to intraperitoneal inoculation but after intracerebral injection they develop a fatal encephalitis. After repeated passages through mice by intracerebral inoculation, the virus becomes altered so that it usually causes no apparent infection when inoculated intraperitoneally into rhesus monkeys. If given intracerebrally, however, it causes a fatal encephalitis in the monkey. Virus so altered is commonly termed "neurotropic" virus, in contrast to the "viscerotropic" natural virus which, however, also has neurotropic properties. Intraperitoneal injections of neurotropic virus into normal mice usually cause no evident infection, but if the brain has been previously injured, as by intracerebral injection of sterile starch paste, it will cause an encephalitis. The hedgehog is highly susceptible. Other rodents and other species of monkeys may be infected by intracerebral inoculation.

By passage of the natural virus through tissue cultures and chick embryos for a long period, a similar but more marked attenuation was secured. This strain (17 D) shows little tendency to cause any evident infection when inoculated either into monkeys or man, although it will still cause encephalitis on intracerebral injection into mice. It does cause an inapparent infection, however, as it usually produces an immunity, and has been used extensively as a vaccine for human beings.

The virus is present in the blood in man during the first three days of the disease (rarely the fourth) but not later. The concentration may be enormous, even ten billion infectious doses per ml. It is present in the blood of monkeys in high concentration. It is also present in the liver and in other tissues in fatal cases.

ANTIBODIES. Following recovery, specific antibodies, particularly neutralizing antibodies, are present in the serum. This was demonstrated on human volunteers by Marchoux et al. (1903). Later monkeys were used, and at present white mice, according to the method of Theiler, as modified by Sawyer and Lloyd (1931). Six mice are used for each serum to be tested. These are given a preliminary intracerebral injection of 0.03 ml. of starch paste. A 15 to 20 per cent suspension of the brain of a mouse dying from infection with the neurotropic strain of virus is prepared in physiologic salt solution containing 10 per cent of normal serum. After the coarse particles are removed by centrifugalization the suspension is mixed with an equal volume of serum, and 0.5 ml. of this mixture is injected intraperitoneally (Sellards). Control series with normal serum and known immune serum should be carried out. The presence of antibodies is demonstrated by the survival of at least four of the six mice which were injected with the serum under investigation, provided that at least five of six unprotected mice die within 5 to 10 (usually 6) days. This procedure has been widely used on thousands of individuals in epidemiologic studies, and misleading reactions indicating immunity have been very rare.

Antibodies appear early, even before the virus has disappeared from the blood, and may be present in fatal cases. The antibodies following natural infection persist for indefinite periods, in individual cases at least 75 years. They are generally regarded as indicating an effective immunity. They also appear after successful vaccination, but it is not certain that there is a precise parallelism between their titer and the degree of immunity present.

EPIDEMIOLOGY. Yellow fever appears in two types, the classical urban type, characterized by explosive and often extensive epidemics limited largely to densely populated areas, and a sporadic, endemic, or "jungle" type. The difference is purely in the mode of dissemination. The same virus is found in both, and the clinical features and pathologic lesions are identical.

In the urban epidemics the virus is transmitted by the *Aëdes aegypti* mosquito. This was suggested by Finlay on the basis of clinical observations, and demon-

strated conclusively by the U. S. Army Yellow Fever Commission (Reed, Carroll, Lazear, and Agramonte) in Cuba in 1900. It was shown that the mosquito becomes infected by biting a patient during the first three days of the disease, but that it is unable to transmit the infection until after a period of about 12 days has elapsed (the extrinsic incubation period of Corré). The mosquito then remains infectious for the remainder of its life.

The virus is present in the mosquitoes during this incubation period; this fact can be demonstrated by grinding up the insect and injecting it into monkeys or mice. The virus multiplies in the mosquito, but there is no evidence that it goes through any developmental cycle. The length of the incubation period depends upon the environmental temperature, which determines the rate of multiplication, varying from five days at 37° C. to three weeks at 20° C. The virus becomes highly concentrated in the mosquito (1 to 1,000,000), and a single bite suffices to convey the disease. Infection is not transmitted to the next generation; the mosquito cannot serve as a reservoir of infection. The mosquito suffers no evident injury from the infection, and no change can be detected in its tissues.

Under experimental conditions yellow fever has been transmitted (by the bite) by at least 20 other species of mosquitoes, belonging to Aëdes and to several other genera, and by several species of ticks. Other arthropods may harbor the virus without transmitting it by the bite. There is no evidence, however, that any other species plays a significant role in the urban epidemics, although it is conceivable that they might do so. Thus far no arthropod vector has been shown to serve as a reservoir of infection.

Before the advent of vaccination, the incidence of accidental infection among laboratory workers was very high. There is strong reason to believe that infection can be acquired through the apparently unbroken skin, by handling infected blood or tissues, and probably through the conjunctiva and respiratory passages. Some infections have been attributed to handling desiccated powdered virus. Infection is not acquired by direct contact with patients or with fomites.

Jungle Type of Yellow Fever. Extensive studies over the past decade have shown that yellow fever occurs as sporadic cases and occasionally in small epidemics in isolated outlying districts over vast areas in the Amazon basin in Brazil and in Colombia, in which *Aëdes aegypti* is not found. For the most part, the disease affected men who were laborers in the jungle, sparing the women and children except when the families lived in or adjoining the jungle. This suggested that transmission must be brought about by species of mosquitoes which infest the jungle rather than the settlements. As the result of a vast amount of effort, yellow fever virus was demonstrated under natural conditions in two such species, *Aëdes leucocelaneus* and *Haemagogus capricornii*.

In jungle districts the incidence of the disease in man seemed too small to furnish an adequate reservoir of infection, and some of the jungle fauna have been suspected. No mammal has yet been found naturally infected, but there is reason to believe that monkeys may constitute the reservoir. Soper showed that these animals are highly susceptible, and in a significant number of those captured he was able to demonstrate the presence of neutralizing antibodies in the blood. Findlay (1935) suggested that the hedgehog may play a similar role in African yellow fever, and he also demonstrated protective power in the blood of monkeys captured in regions in which the disease is endemic.

Vaccination. The purpose of vaccination is to produce an inapparent infection by means of an attenuated virus which is still capable of stimulating an effective immunity. In the first attempts at large-scale vaccination in Africa, Sellards and Laigret (1932) used the

neurotropic mouse-brain virus. This "is virulent and produces an enduring immunity with the risk of a small proportion of serious reactions" (Sellards). Later, immune serum was added for further attenuation. Subsequently the attenuated cultural strain (17 D) was used, and vaccination with this material without the addition of any serum has practically replaced all other methods. This strain is lower in virulence, and serious reactions are extremely rare. It has been used on a huge scale in Brazil, and also in the armed forces, and appears usually to yield a satisfactory degree of protection in a large proportion of the subjects. Protection is somewhat less certain, however, and probably of shorter duration than that obtained with more potent types of vaccine. Cases of yellow fever have occurred in individuals so vaccinated.

The jaundice following yellow fever vaccination which occurred in members of the U. S. Army early in World War II must be regarded as an unfortunate accident, due to the presence of infectious hepatitis virus in the serum used in preparing the vaccine. This had nothing to do with the yellow fever virus and has not occurred since the use of serum has been abandoned.

Neutralizing antibodies appear in the serum of a large proportion of vaccinated individuals, and are regarded as an approximate measure of immunity.

Prevention. For the prevention of urban epidemics, sanitary measures designed to control *A. aegypti* mosquitoes and particularly to eliminate their breeding places are of great importance. Where such measures have been carried out effectively and persistently, the disease has been virtually eliminated. For the jungle type, no practicable measures of vector control are available, and vaccination is the only effective procedure. For laboratory personnel, effective vaccination is imperative, and has practically eliminated the accidental infections which formerly were so numerous.

Dengue. This disease is caused by a filtrable virus which is transmitted by certain species of Aëdes (Stegomyia), in which the virus is propagated.

Its onset is extremely abrupt. A short initial fever of three or four days is followed by a rapid fall of the temperature, a short remission, and then a short terminal fever—saddleback chart. The end of the primary fever is often characterized by critical sweats, diarrhea, and epistaxis. The striking eruption, which has given it the name *bouquet,* appears about the time of the remission. Severe pains in the back, joints, and postorbital muscles make the name given by Benjamin Rush appropriate—breakbone fever. The subsequent neurasthenia and physical weakness are very striking.

Arthropod Vector. Siler (1926) transmitted the disease by *Aëdes aegypti* in 47 out of 111 human experiments. The cycle in the mosquito requires 11 to 14 days, and the infecting feeding must take place during the first three, or possibly five days (Blanc) of the disease in man. The mosquito retains infecting power throughout its life—174 days (Blanc).

Virus of Dengue. Virus-containing serum remains infective for two or three months if kept in sealed tubes and in the dark. The virus is destroyed by a temperature of 50° C. for 30 minutes. Blanc has found that desiccated and refrigerated serum would remain virulent only 95 days. It has been found possible to transmit the virus to monkeys, producing after five days of clinically inapparent infection an infecting serum which will cause the disease in man, through transmission by Aëdes fed on monkey blood. Cultivation on the chorioallantoic membrane of chick embryos has been reported.

Neutralizing antibodies have been demonstrated after recovery. Immunity is variable and relatively brief.

Relationship to Yellow Fever. Although epidemiologically and clinically dengue shows some similarity to yellow fever, the viruses are antigenically entirely different. Neither disease affords any protection from the other.

Colorado Tick Fever. This is a benign but painful infectious disease occurring in Colorado and neighboring Western states, which was first differentiated from mild Rocky Mountain spotted fever by Becker in 1930. It closely resembles dengue in its clinical symptoms, in a leukopenia (2000 to 3000), in the "saddle-back" temperature curve and in the invariably favorable outcome. By means of experiments on human volunteers, Florio et al. (1944, 1946) showed that the active agent is present in the blood during the acute stage and that recovery is followed by an immunity. They established the infection in golden hamsters by serial passages. The agent was filtrable through a gradacol membrane with a pore diameter of 24mμ, and it is therefore one of the smallest of the viruses. Antigenically it is entirely unrelated to dengue. Epidemiologic evidence suggests that it is transmitted by the wood tick, *Dermacentor andersoni,* but attempts to convey the infection experimentally by this arthropod have been unsuccessful.

Rift-Valley Fever (Enzootic Hepatitis). This is an epizootic disease of sheep, caused by a filtrable virus described by Daubney, Hudson, and Garnham in 1931. During the study of a severe epidemic in Kenya, British East Africa, it was noted that the native shepherds, and later the Europeans, in contact with the affected animals developed a short illness resembling dengue. These investigators also produced the disease in a volunteer by inoculation with the blood of an infected sheep. Since that time a number of laboratory workers in this country and abroad have contracted the disease while studying the virus.

The disease can be transmitted to cattle (a small epidemic of natural infection is recorded), goats, certain species of monkeys, and to rats, mice, and other small rodents. Guinea pigs and rabbits, however, appear to be resistant. The infection can be produced by application of the virus to scarified skin, the conjunctivae, or nasal mucosa, as well as by subcutaneous or intraperitoneal injection. There is some evidence to suggest that mosquitoes of the genus *Mansonia* act as vectors. The characteristic pathologic lesion in lambs, and also in inoculated mice, is a marked focal necrosis of the liver. Stained sections show acidophilic intranuclear inclusions in the hepatic cells.

Mackenzie and Findlay (1936) obtained a "fixed" neurotropic strain of Rift-Valley fever virus by the injection of immune serum into an animal prior to an intracerebral inoculation of virus. Some of the nerve cells showed intranuclear inclusions similar to those produced by the neurotropic type of yellow fever virus. They suggest that this restraining action of specific antibodies may produce neurotropic variants of other viruses, and that the occurrence of nervous sequelae in virus infections in man may be explained in a similar way.

Virus of Rift-Valley Fever. The virus occurs in the blood, liver, spleen, and other organs early in the disease in both animals and man. It will pass through Berkefeld candles *N, V,* and *W;* and Broom and Findlay have estimated its size at from 23 to 35mμ by filtration through collodion membranes. Mackenzie has cultivated the virus in a medium of chick embryo and Tyrode's solution. It can be preserved in blood containing 0.5 per cent phenol for months in the icebox.

Immunity. The serum of convalescent animals and man contains virus-neutralizing antibodies for several months after recovery, but the immunity is not permanent. Broom and Findlay have demonstrated complement-fixing antibodies which are apparently specific for Rift-Valley fever, and which they found to persist for at least six months. Francis and Magill (1935) have shown that the inoculation of insusceptible animals such as the rabbit also results in the development of virus-neutralizing antibodies.

Diagnosis. The diagnosis of Rift-Valley fever depends upon the demonstration of the virus in the blood by mouse inoculation in early cases, or of virus-neutralizing antibodies later in the disease. The clinical picture resembles that of influenza, dengue, or yellow fever. The virus of influenza, however, is not present in the blood; and the viruses of dengue and yellow fever are not pathogenic for mice on intraperitoneal inoculation. There is no cross immunity with the viruses of dengue or yellow fever.

Phlebotomus Fever (Pappataci Fever, Sandfly Fever). This disease resembles dengue, influenza, and similar infections, but it is of short duration—usually only three days. It is due to a filtrable virus which is present in the blood during the first 24 to 48 hours. It is transmitted by a moth midge or sandfly, *Phlebotomus papatasii* (possibly other species also), in which a developmental period (extrinsic incubation) of from six to eight days must elapse before it becomes infective. The virus has been shown to be transmitted to a second generation of these flies, which apparently constitute a reservoir of the infection. (See p. 684.)

Bacteriophage

Bacteriophage is a filtrable particulate substance which causes lysis, or dissolution of susceptible bacteria. It increases in quantity in the process and can be transmitted indefinitely from one culture to another. There are a great many different varieties or "species" of bacteriophage which can be differentiated by appropriate tests.

Twort, in 1915, while working on the filtrable viruses, observed a "glassy degeneration" of certain colonies of cocci in which no intact organisms were demonstrable in smears. Inoculation from these areas into normal colonies produced similar areas. This lytic agent was filtrable and could be transmitted serially in cultures. In 1917 d'Herelle discovered that sterile filtrates from the feces of a patient having Shiga bacillus dysentery contained a substance which would, after a preliminary period of incubation (4 to 24 hr.), inhibit and finally dissolve the organisms in an actively growing culture of the Shiga bacillus. He also found that filtrates from this lysed culture acted similarly on a fresh culture even in minute amounts, and that the lytic principle could be propagated indefinitely in Shiga bacillus cultures.

The nature of this lytic substance has been much disputed. D'Herelle believed that it is a minute organism living as a parasite on susceptible bacteria. Most students now regard it as a filtrable virus.

Demonstration in Cultures. Bacteriophage can be obtained easily from feces or sewage. It is often present in water and soil and has been isolated from pus, infected urine, and other substances. It is sometimes present in laboratory cultures in a latent or even in an active form. After isolation by filtration its potency can be increased by repeated inoculation into susceptible young cultures. Old or dead cultures are not affected. It is never demonstrable except in conjunction with actively growing young cultures.

Its presence may be demonstrated in either fluid or solid cultures. Inoculation of a suitable broth culture causes a partial or complete clearing of the turbidity in from 4 to 24 hours. The potency of the bacteriophage preparation can be measured by adding varying amounts to a fresh culture. In some cases as little as one part of the filtrate in a billion parts of culture will effect lysis. If transplants of such a mixture are made to an agar plate shortly after inoculation, a surface growth is obtained which is pitted with clear glassy areas or plaques. These plaques may occur in the center or around the edges of discrete colonies, giving the culture a moth-eaten appearance. Smears from these areas

show only an amorphous debris. D'Herelle referred to these plaques as colonies of bacteriophage.

When lysis is complete, no growth is obtained in subcultures. In most cases, however, a few organisms are resistant and after prolonged incubation resume multiplication. Such survivors are specifically resistant to the strain of bacteriophage present in the culture, but usually are susceptible to other types of bacteriophage. They frequently show other variations from the original culture. Virulence may be enhanced or diminished. S forms may be changed to R forms, and mucoid types may develop.

Virus of Bacteriophage. The size of the virus probably varies in different varieties. Staphylococcus phage has been reported to have a diameter of 90mμ on the basis of filtration and sedimentation tests with concentrated suspensions, whereas diffusion experiments with dilute suspensions indicate a diameter of only 10mμ. Bacteriophage may withstand heating to 75° C. for one-half hour and resists desiccation. According to d'Herelle its resistance in general is greater than that of vegetative bacteria and less than that of spores.

Specificity. A bacteriophage is usually (though not always) specific for a certain species of bacterium. It may act on closely related species. Sometimes its activity is limited to a certain strain of a species. Adaptation to other organisms may occur to some extent. A single species of bacterium may be susceptible to several distinct bacteriophages.

Bacteriophage is antigenic. Since it cannot be obtained free from lysed bacterial bodies, antisera also contain ordinary bacterial antibodies. After absorption of such sera with the bacteria themselves, however, a substance remains which will inhibit the action of the homologous bacteriophage. This antibody is specific for the strain of bacteriophage used in its production, irrespective of the bacterial substrate upon which it was propagated.

Bacteriophage may propagate in cultures in which the bacteria are not lysed. Filtrates from such cultures will cause lysis of other strains of the same species of bacterium which have not acquired resistance to the bacteriophage. Such bacteria may be regarded as carriers.

Bacteriophage exerts little or no action on bacteria *in vivo*. Attempts to utilize it for therapeutic purposes have given disappointing results, although some have claimed good results from topical applications in localized septic infections, and in colon bacillus infections of the urinary passages.

CHAPTER 9

Medical Mycology

Classification

The fungi belong to a group of plants known as *Thallophyta.* They are filamentous plants which do not develop a complex body and do not develop root, stem, or leaf. There are two large subdivisions of the Thallophyta: algae, containing chlorophyll, and fungi, lacking chlorophyll. Three large groups of fungi may be recognized:

1. *Schizomycetes,* or bacteria.
2. *Eumycetes,* or "true fungi," which may be defined as unicellular or multicellular plantlike organisms, usually larger than bacteria, with definite cell walls; which multiply by cell fission, budding, or usually by means of sexual and asexual spores. This group includes the pathogenic fungi.
3. *Myxomycetes,* or slime molds, which are multinucleated, naked masses of protoplasm. No species is pathogenic for man.

The classification of the fungi is difficult because of the great number of species (upwards of 100,000), the complexity of the life cycles of many fungi, the creation of new generic and specific names for fungi already validly named, and failure on the part of authors of new species names properly to evaluate the great frequency and the true significance of the variability of fungi. Consequently there are discrepancies between the taxonomic systems presented by different investigators, depending on the mycologic experience of the writer and his interpretations of minor variations. The classification of the fungi will remain difficult because of the first two items mentioned, but it can be partially clarified by acquisition of further knowledge of life cycles and a more realistic interpretation of the common occurrence of variation and mutation.

A Classification of Fungi of Medical Importance

Class	*Genus*	*Species*
Phycomycetes	Mucor	*M. mucedo*
	Absidia	*A. corymbifer*
	Rhizopus	*R. niger*
		R. parasiticus
	Coccidioides	*C. immitis*
Ascomycetes	Allescheria	*A. boydii*
	Trichosporum	*T. giganteum*
	Piedraia	*P. hortai*

Class	Genus	Species
Fungi Imperfecti	Sporotrichum	*S. schenckii*
	Blastomyces	*B. dermatitidis*
		B. brasiliensis
	Rhinosporidium	*R. seeberi*
	Histoplasma	*H. capsulatum*
	Candida (Monilia)	*C. albicans*
	Cryptococcus	*C. neoformans*
	Aspergillus*	*A. niger*
		A. fumigatus
	Penicillium*	*P. crustaceum*†
		P. notatum†
	Scopulariopsis	*S. brevicaule*
	Malassezia	*M. furfur*
	Microsporum	*M. gypseum*
		M. audouini
		M. canis
	Trichophyton (Including ectothrix, endothrix, and neo-endothrix species. Achorion and Endodermophyton are included in this genus.)	*T. mentagrophytes*
		T. violaceum
		T. tonsurans
		T. sabouraudi
		T. schoenleini
		T. concentricum
	Epidermophyton	*E. floccosum*
	Phialophora	*P. pedrosoi*
		P. compactum
		P. verrucosa
	Madurella	*M. mycetomi*
	Indiella	*I. mansoni*
	Monosporium	*M. apiospermum*
	Cephalosporium	*C. recifei*

Diseases caused by fungi are known as "mycoses." The terminology of the mycoses varies considerably with different authors, but fairly well-recognized terms are the dermatophytoses, or ringworm affections, otomycoses, onychomycoses, and maduromycoses. Again, the name of the affection may be taken from the fungus concerned in the cause, as sporotrichosis, blastomycosis, or aspergillosis. However, there is also great divergency of opinion in such classification. Thus, Brumpt (1936), under the term "blastomycosis," includes only the diseases caused by the budding fungi (*Saccharomyces, Mycotorula,* and *Torula*) or those presenting the form of yeasts (blastospores); and he definitely excludes the organisms of the nonbudding fungi. In the United States, however, blastomycosis refers to infection caused by *Blastomyces.*

Morphology of Fungi

The fungus plant may structurally be divided into two parts, the vegetative part (thallus or mycelium), and the reproductive part (spores).

*Conant (1945) places *A. bonffardi* and *P. mycetogenum* in the class *Ascomycetes.*

†*P. crustaceum*, although not proved pathogenic to man, is included because of its ubiquitousness, and *P. notatum* is included because of interest in its production of penicillin.

The thallus or mycelium is composed of a mass of individual strands or filaments called "hyphae," which may or may not be divided into cells by cross walls, or septa. A mycelium composed of septate hyphae is called a "septate mycelium," whereas one which lacks septa is termed a "coenocytic mycelium." The presence or absence of septa is of taxonomic importance. The class *Phycomycetes,* for example, is characterized by members possessing a coenocytic mycelium.

Some hyphae contain cellulose, others chitin. In some fungi the mycelium becomes packed as a hard mass, containing reserve food material, and known as "sclerotia." Similar dense structures are produced by *Piedraia hortai,* causing a disease of the hair, and by the fungi producing granules in the mycetomas. Ergot is the sclerotium of a fungus (*Claviceps purpurea*) attacking the grain-bearing heads of rye, and is of importance medically. In the Middle Ages there were great epidemics of ergot poisoning.

Other variations of hyphae which are of morphologic importance in the classification of fungi are spiral hyphae, which are simply coiled, corkscrew-like filaments, and racquet hyphae, which are hyphae composed of club-shaped cells, the clubbed end of one cell being attached to the small end of an adjacent cell. In some species of fungi hyphae occasionally develop unilateral projections from a terminal hyphal cell which resemble a broken comb. These are called "pectinate bodies."

The reproductive portion of the fungus plant is made up of spores. Spores may have a sexual origin, resulting from the fusion of two cells with subsequent nuclear fusion, or they may be asexual. Perfect fungi are those which reproduce by means of sexual spores, whereas the imperfect fungi (*Fungi Imperfecti*) reproduce by asexual spores.

A spore may be simply defined as a cell which may or may not separate from



(A) Coenocytic hyphae. (B) Septate hyphae. (C) Racquet hyphae. (D) Pectinate hypha. (E) Spiral hypha.

the hypha but which is capable of germinating and reproducing the parent cell of the hypha. A specialized hypha to which spores are attached is termed a "sporophore." Occasionally specialized cells are germinated from the sporophore from which spores are produced. These specialized cells are called "sterigmata."

The simplest type of spore development is that in which cells are formed by the septation of hyphal cells which in turn develop new hyphae. These simple forms of spores are called "arthrospores" or "thallospores." Sometimes thallospores are formed by the concentration of the protoplasm of the hypha and thickening of the cell membrane, and are purely resting spores. This type of thallospore is termed a "chlamydospore." Thallospores which develop by budding from the end or side of the parent hyphal cell and which may, in turn, produce another bud or hypha without becoming detached are called "blastospores." The buds of yeast cells are familiar examples. Spores originating asexually from the hyphal cell by a process of budding, septation, or abstriction are generally called "conidia."

Fuseaux or macroconidia are large, elongated, chambered bodies resembling chlamydospores, arising singly or in clusters from hyphal cells, and divided into segments by transverse septa.

The spores which have been described above are all examples of asexual exospores (free-borne from the mycelium). A special class of sexual endospores which are characteristic of the class *Ascomycetes* are termed "ascospores." Ascospores are produced within a membrane or sac called an "ascus," and the number of spores is usually limited to two, four, or eight, and is constant for the particular species producing them.

"Basidiospores" are spores which develop after nuclear fusion and subsequent reduction division on a special type of sporophore known as a "basidium." The basidiospore is characteristic of the class *Basidiomycetes* (the large, fleshy fungi or mushrooms). What we call a mushroom consists only of the spore-bearing part of the plant which is above ground. An extensive mycelium exists underground.

"Zygospores" and "oöspores" are sexual spores produced by the fusion of two cells.



Spore types. (A) Blastospores. (B) Chlamydospore. (C) Conidia. (D) Budding yeast cell—blastospores. (E) Conidiophore with sterigmata bearing conidia. (F) Ascus containing ascospores. (G) Basidiospores. (H) Zygospore. (I) Fuseaux or macroconidia.

Examination of Fungi. The identification of fungi is to a greater extent dependent on morphologic characteristics than is the identification of bacteria. Fungi possess structures which are characteristic of the genus and species. These structures have names and consequently the worker must acquaint himself with a new vocabulary. This, apparently, is the major difficulty of the inexperienced technician who is confronted with the problem of diagnosis of an unknown fungus. Actually, recognition and identification is relatively simpler than the same procedure involving bacteria. We are not confronted, for the most part, with biochemic reactions and the wide variations of structure and physiology to which bacteria are subjected.

For observing the morphology of molds a plate culture is in many cases to be preferred to a test-tube slant. Petri-dish cultures of the highly pathogenic fungi, however, are not recommended. Easily disseminated spores can be inhaled and numerous laboratory infections have been reported. Frequently, with experience, the gross appearance of the colony is sufficient to enable the worker to arrive at a tentative diagnosis.

It is often possible to place the unknown mold in the class *Phycomycetes* or in the *Fungi Imperfecti* by gross characteristics. With those species which belong to the *Fungi Imperfecti* and to the *Ascomycetes* the plant mass is relatively compact, with short aerial filaments of mycelium, and the surface, which may be brightly colored, is often thickly covered with spores. The entire colony resembles the nap of velvet. With fungi of the class *Phycomycetes,* the mycelium is coarser and looser in structure. The spore heads and the aerial filaments are sometimes dark brown, gray, or black. The plant resembles cotton wool.

Many fungi secrete fluid which collects in droplets on the surface of the colony. The droplets of moisture may be colored by various pigments excreted by the organism. These droplets may be mistaken by the beginner for spores and other similar objects.

A hand lens or the low power of the microscope may be used in examining the surface of the colony. If a dissecting microscope is available this is the instrument of choice. After the upper side of the colony has been carefully examined it is important to turn the plate over and observe the gross characteristics of the mold from this angle. The color of the embedded mycelium should be carefully noted, and the medium examined for the presence of any diffusable pigment. These pigments may at times act as indicators, being one color at an alkaline pH and another color at acid pH.

For finer details medium-powered microscopic examination is necessary. A small portion of the colony is removed with a sharp, stiff needle. Depending on the fungus, it is sometimes advisable, in addition, to remove a piece of the medium which contains the embedded mycelium. The specimen is teased out with needles in a drop of saline, sodium hydroxide solution, lactophenol, or other mounting fluid, and a coverslip placed over the preparation. The specimen is then carefully examined for the presence or absence of septa in the mycelium, the structure of the sporophores and spores, etc.

Cultivation of Pathogenic Fungi. Generally, fungi are well able to tolerate highly acid media. Not all pathogenic fungi do so, however, and it is therefore advisable to cultivate an unknown fungus on a variety of media. Dextrose tartaric acid medium and Sabouraud's medium will support the growth of most fungi. Great care must be taken to maintain the sterility of the medium. Molds may be isolated by simply exposing a

medium plate to the air for a few minutes. These contaminants will prove most annoying. Fungi are, in general, slow growers and several weeks incubation may be necessary before growth of some pathogens is apparent.

Since most molds grow well on media with an acid reaction, we may adjust the pH to 5.2 or even lower to permit the growth of fungi but inhibit to a great degree bacterial development.

Glycerin agar, bread paste, or potato media are all suitable, but the standard medium is that of Sabouraud.

Sabouraud's Conservation Medium

(for preserving stock cultures)

Peptone	30 Gm.
Agar (shred)	15 Gm.
Tap water	1000 ml.

Sabouraud's Differentiation Medium

(two of these are used, a maltose medium and a glucose medium)

Glucose	40 Gm.
Peptone	10 Gm.
Agar	15 Gm.
Tap water	1000 ml.

The maltose medium is made by substituting the maltose for glucose in the above formula.

In each case the ingredients are added to the water and all placed in a cold autoclave and the pressure allowed to rise in both outer and inner jacket simultaneously until it has reached 15 pounds. The autoclave is then shut off and allowed to cool down slowly. When cooled the medium is filtered through cotton, tubed, and then sterilized as above. Upon removal from the autoclave the tubes are slanted and allowed to cool in the slanting position.

The medium is not titrated or the pH adjusted in any way, Sabouraud claiming that the addition of either acid or alkali spoils the medium.

Some mycologists claim that the Sabouraud medium contains too much sugar and that this interferes with growth and morphology. The monosaccharides seem to be more objectionable than the polysaccharides. Besides potato slants, such solid media may be made from carrots in particular, but any fruit or vegetable may be similarly prepared.

Falchi's Carrot Agar

(recommended for demonstrating the color characteristics of a colony)

Carrots	500 Gm.
Peptone	10 Gm.
Agar	20 Gm.
Water	1000 ml.

The vegetables are washed, peeled, and cut into small pieces. To them are added 700 ml. water and the mixture boiled down to 500 ml. It is then filtered through paper. The agar is dissolved in 500 ml. water by heating. The vegetables and the agar are mixed, placed in tubes, and autoclaved for 20 minutes at 15 pounds pressure. The tubes are slanted and allowed to cool and harden.

In cultivating molds, small Erlenmeyer flasks or Petri dishes, containing about ¼ inch of medium on the bottom, will be most suitable for the development of colonies. It must again be emphasized, however, that Petri dishes are not recommended for colonial

study of the highly pathogenic fungi. Inhalation of spores has resulted in laboratory infections. In order to isolate fungi from the dermatophytoses, hairs or scales are placed in a sterile Petri dish and cut into small fragments with a sterile knife. These pieces are covered with 70 per cent alcohol for two to eight minutes, then a wire loop is moistened from the surface of the agar, a fragment is touched with the loop and when it adheres it is transferred to the surface of the medium. Four or five inoculations are made on the surface, and from a suitable colony the fungus should be subcultured to a fresh culture medium.

Plaut recommends that mold material be placed between two glass slides. One edge is sealed with wax and the preparation is placed in a moist chamber for four to seven days. From the developing fungus growth, medium in a flask or Petri dish is inoculated. A Petri dish containing several layers of thoroughly moistened filter paper in top and bottom makes a satisfactory moist chamber.

One method of identification of molds consists in filling the concavity of a hollow slide, which has been flamed for sterilization, with melted Sabouraud's agar or any other suitable medium. The surface is then inoculated with material from the colony to be studied and a flamed (sterile) coverslip applied. In a similar method which was devised for the study of bacterial colonies, Hill used 1-cm. squares of agar, cut out from sheets made by pouring melted agar into Petri dishes. Another method consists in permitting a large drop of melted agar to spread over a sterile coverslip and then inoculating the film of medium and adjusting over a concave slide. These methods are considered excellent for bringing out the mode of fructification of molds which can then be studied satisfactorily with high-power lens. Some important morphologic features, however, may fail to develop under these restricted conditions.

Examination of Skin Scrapings and Hair for Fungi. Dried hairs or scales are immersed for clearing in sodium hydroxide or potassium hydroxide solution in concentration of 10 to 30 per cent. Forty per cent solutions may be used where rapid clearing is desired but this concentration tends to swell and disintegrate the fungi. Clearing of the hairs or scales takes place within five minutes to two hours in 10 per cent potassium hydroxide. The gentle application of heat will accelerate clearing. After clearing, the scales or hairs are examined under low or medium power using a subdued light. The spores or mycelium of the fungus are easily discernible as refractile bodies against a clear, translucent background. When hairs or scales are particularly oily it is advisable first to dip them in either ether or 95 per cent alcohol before clearing to remove the oil droplets which may sometimes be mistaken for spores.

Adamson recommends clearing the hairs or scales in 5 to 10 per cent potassium hydroxide solution, washing in water, and then staining by Gram's method. The fungi are Gram-positive.

Chalmers and Marshall report success with the following technic:

1. Soak scales in 40 per cent potassium hydroxide for a few hours in a watch glass in the incubator at 40° C.
2. Wash in 15 per cent alcohol for 30 minutes.
3. Place scales on a slide, evaporate the alcohol, and dry over a flame.
4. Stain with gentian violet for 20 minutes.
5. Wash and add Gram's iodine for three minutes.
6. Decolorize with aniline oil for 30 minutes.
7. Wash and stain in concentrated alcoholic eosin for one minute.
8. Wash with aniline oil or clove oil.
9. Immerse in xylol and mount in balsam.

Berberian's method is recommended to differentiate fungi within epithelial cells from blood cells, bacteria, and "mosaic fungi."

1. Cover hairs or scales on a slide with 50 per cent glacial acetic acid and dry in the incubator.

2. To defat, clear, and dehydrate, cover the preparation with ether two or three times, 20 to 30 seconds each time. Then flood two times with absolute acetone and follow by flooding with absolute, 95 per cent, 70 per cent, and 50 per cent alcohol.
3. Stain with Martinotti's stain.
   a. Distilled water ..... 75 ml.
   b. Lithium carbonate ..... 0.5 Gm.
   c. Toluidine blue ..... 1.0 Gm.
   d. After the stain dissolves add 20 ml. glycerin and 5 ml. 95 per cent alcohol.
4. Wash with water and differentiate with 0.5 per cent acetic acid.
5. Dehydrate with absolute acetone for two to three minutes.
6. Clear in xylol and mount in balsam.

Various clearing agents have been recommended instead of sodium hydroxide and potassium hydroxide solutions. Priestley suggests lactophenol, which is made up of lactic acid, one part; phenol, one part; glycerol, two parts; distilled water, one part. For thick material Langeron uses chloral hydrate crystals, 40 Gm.; phenol crystals, 40 Gm.; lactic acid (USP), 20 Gm.; sodium salicylate, 10 Gm. Slight heating hastens the process of clearing.

Dodge recommends a 1 per cent aqueous solution of cotton blue, a drop of which is placed on a slide and inoculated with the fungus material. A coverslip is then applied and plain lactophenol is drawn under the coverslip. A piece of filter paper at one end of the mount absorbs the aqueous solution and draws in the lactophenol from a drop on the other side of the square coverslip.

Examination of Sputum and Pus. The suspected sputum, pus, or exudate is placed on a slide and 10 per cent potassium hydroxide or sodium hydroxide solution is added. A coverslip is placed over the preparation and the slide examined under the microscope for the presence of mycelium or spores.

The cotton blue-lactophenol method of preparation and staining is the most satisfactory since it eliminates some of the artefacts so easily mistaken for fungi. The most important artefacts frequently encountered are fat cells, potassium hydroxide crystals, and mosaic structures. Mosaic structures are irregular, mycelium-like structures found in the intercellular spaces. Most mycologists agree that these structures are not fungus mycelium. Davidson and Gregory (1935) state they are cholesterol crystals.

Tissue Sections. For staining fungi in sections of tissue, Busse recommends the following stain:

1. Hematoxylin, 10 to 15 minutes; then wash in tap water.
2. Carbolfuchsin (5 per cent), 30 minutes or overnight.
3. Decolorize in alcohol for a few minutes, then pass through absolute alcohol and xylol, and mount in balsam. The fungi are red.

Phycomycetes

The *Phycomycetes* are sometimes referred to as the lower fungi, as they more closely approach the primitive forms. The *Ascomycetes* and the *Fungi Imperfecti* (*Hyphomycetes*) are classified among the higher fungi. The *Phycomycetes* are not usually regarded as of great importance in human pathology, with the exception of *Coccidioides immitis* which is the cause of coccidioidal granuloma. The majority of *Phycomycetes* likely to be encountered will fall in the family *Mucoraceae*. The *Mucoraceae* are a group of molds, frequently referred to as bread molds, found in the soil, manure, fruits, and on starchy foodstuffs. They all have the same general structure and appearance and are easily differentiated from other groups of molds by the coarse, nonseptate mycelium, by the abundant and loosely meshed

aerial mycelium, and by the lack of distinctive colors, the spores being generally black or brown and the mycelium white or gray.

The best known species is *Mucor mucedo,* so common on horse dung, but it is now denied that this fungus plays any part in human mycoses, although a case of Mucor keratomycosis has been reported, in which this common mold was isolated from a corneal lesion.

Mucor Corymbifer (Absidia Corymbifer). The sporangium of this fungus averages 50 to 70μ in diameter, with smaller ones from 10 to 20μ. The spores are spherical and measure 3 to 5μ in diameter. *Mucor corymbifer* grows well on Sabouraud's medium. It has been reported in a number of instances as the cause of nasal, pulmonary, and auricular mycoses (Brumpt, 1936).

Rhizopus. Two species of this genus, *R. niger* and *R. parasiticus,* have been regarded as possibly pathogenic. The former was isolated from rare cases of "black tongue," and the latter from the sputum of a patient believed to have pulmonary mycosis. Vuillemin and others doubt the relationship to "black tongue" which is now regarded as a symptom of a vitamin deficiency. The columella of *R. niger,* after the dehiscence of the sporangium, is mushroom shaped. The sporangia, when mature, have a black color.

Coccidioidal Granuloma. This disease may occur with nodular, verrucous, ulcerative, or papillomatous lesions of the skin, large saclike subcutaneous abscesses, and sometimes with involvement of the bones, joints, and viscera. The skin lesions often resemble those of tuberculosis with the presence of cold abscesses. Coccidioidal granuloma, however, rarely starts as a skin affection. The usual course of the disease is that of an upper respiratory febrile infection with a cough that ordinarily clears up in a week or so (San Joaquin Valley fever), but may progress and clinically resemble tuberculosis, with night sweats and mucopurulent sputum. The period of incubation is about 12 days. Allergic manifestations, especially erythema nodosum and eosinophilia, occasionally occur after the subsidence of the fever. A rise in blood sedimentation rate, which falls after disappearance of fever, is noted. A continued elevation suggests a progressive coccidioidomycosis.

The first case, affecting a Brazilian soldier, was reported from Argentina in 1892



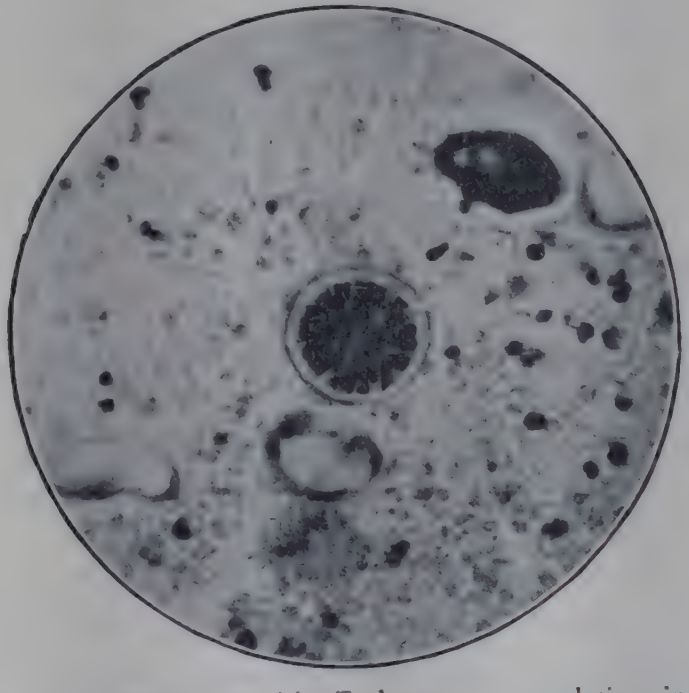
(Left) Mucor sp. (Right) Rhizopus sp.

by Wernicke. Later his assistant, Posadas, made thorough studies of the causative organism, including animal inoculations. Posadas first regarded the parasite as a *Coccidium*. The name *Coccidioides immitis* was given to it by Rixford and Gilchrist, and the parasite was later shown to be a fungus.

The mycosis was first known in the United States in California. It is now recognized to be prevalent throughout the arid regions of the southwestern portion of this country.

Coccidioidal granuloma has been reported from Mexico, Uruguay, and Argentina.

Dickson (1938) has reported that valley fever or desert fever of the San Joaquin Valley is an acute coccidioidal infection. The lesions of the skin resembled erythema nodosum, and there was usually pulmonary involvement. He showed that the infection was caused by the inhalation of the chlamydospores. A great majority



Coccidioides immitis. Endogenous sporulation in center. (Courtesy, Commander H. E. Ragle, Medical Corps, U.S.N.)

of those infected promptly recovered. Infection has occurred in laboratory workers from inhalation of dust from dried cultures.

The fungi occur either isolated or in giant cells. It is the tendency to metastasis through blood and lymph channels that explains the seriousness of the disease. The differentiation from tuberculosis, syphilis, and other fungus infections is made by finding the nonbudding cysts, with endospore formation. A wet preparation is entirely satisfactory for examination. In tissues, more or less round, cystlike structures occur, varying from 4 to 80μ in diameter, containing endospores and having a thick, doubly contoured capsule. These rounded elements never show budding in tissues. Cultures of the organism on artificial media develop grayish-white colonies appearing in 4 to 17 days on malt extract agar, later becoming cottony and brown with an abundance of chlamydospores (4 to 7μ). The hyphae are

about 3μ wide. MacNeal and Taylor have noted endospore formation in anaerobic cultures. Inoculation of monkeys, rabbits, guinea pigs, and mice with cultures gives rise to the same cystlike structures, with endogenous spores, as noted in human tissues.

Complement-fixation reactions with antigens prepared from cultures of the infecting organisms have been obtained. An allergic skin reaction has been described from the injection of such an antigen.

Smith, who tested many thousands in the armed services, reports positive reactions to coccidioidin in cases of coccidioidal granuloma, but no reaction to the antigen blastomycin. In one case of Gilchrist's disease, only a moderately positive reaction was obtained with blastomycin and no reaction with coccidioidin or sporotrichin. Smith believes that coccidioidin is of value in denying or confirming the presence of the corresponding disease.

Hurwitz, Young, and Eddie have reported positive coccidioidal reactions in patients having no known coccidioidal infection (10 per cent). Individuals with positive coccidioidin reactions may show areas of calcification in roentgenograms of the chest.

Farness and Wooley demonstrated that 90 per cent of school children in a district in California in which coccidioidomycosis was prevalent all gave a positive reaction to coccidioidin, showing that the test there would be valueless. In New York, however, where coccidioidomycosis is rare, the test might be of value.

Kessel believes that the skin test can be used as an aid to diagnosis, but probably no more reliance can be placed on it than is usually given to the tuberculin test. He thinks that tuberculosis patients usually give negative reactions. Nevertheless, further careful studies and confirmation of all this work is necessary before it can be regarded as conclusive.

Very little is known as to the source of infection of coccidioidal granuloma, but the great resistance of the cultural spores and the occurrence of laboratory infections have suggested the inhalation of dried spores as the usual mode of inoculation rather than cutaneous entrance from dirt or plant material (thorns or prickles). Stewart and Meyer, and Smith and Emmons have reported the isolation of the fungus from the soil.

It has been suggested that rodents may constitute a reservoir of coccidioidomycosis. Emmons and Ashburn (1942) isolated *Coccidioides immitis* from the lungs of 24 of 163 rodents (species of *Perognathus, Dipodomys,* and *Citellus*) trapped in southern Arizona. A related fungus, *Haplosporangium parvum,* was isolated from 98 animals of this series. Gross pulmonary lesions were found in some of the animals and only microscopic lesions in others. Stiles and Davis (1942) report an increase in infection of the lower animals not only in wild rodents but dogs, cattle, and sheep.

The outlook is generally unfavorable in the generalized form of the disease and death usually occurs within three to four years. In 24 cases collected by MacNeal and Taylor there were only two recoveries. A few patients have recovered following drainage or excision of local lesions. With the recognition of milder forms and

arrested cases of coccidioidal granuloma the mortality rate is estimated at about 40 per cent. Primary coccidioidomycosis is benign and very few cases progress.

Ascomycetes

Among the higher fungi are included members of the classes *Ascomycetes* and *Fungi Imperfecti*. The *Ascomycetes* are characterized by a special type of sporangium called an "ascus" (little sac). In the young ascus there is a nuclear fusion, and the fusion nucleus, by three successive divisions, typically results in eight uninucleated ascospores. In some forms, however, some of the nuclei degenerate and only two or four ascospores may result, while in others additional mitotic divisions yield 16 or more.

The yeasts which reproduce typically by budding, but produce ascospores, are included in this class. Other budding forms may fail to show ascospore formation (asporogenous yeasts). In some *Ascomycetes* there is a well-developed thallus, with septate hyphae (the hyphae of the *Phycomycetes* are coenocytic). In the yeasts only a sprout mycelium is known.

The genus *Allescheria,* frequently causing mycetoma, and the genera *Trichosporum* and *Piedraia,* causing piedra, are included under the *Ascomycetes*. Conant (1945) includes certain species of *Aspergillus* and *Penicillium* in the *Ascomycetes*. These two genera, however, will be discussed in more detail in this section under the *Fungi Imperfecti*.

Piedra (Trichosporosis). This is a fungus disease of the hair, in which small nodules form along the hair shafts. Occurring on the beard the disease is known as piedra nostras. The nodules are about the size of the nits of head lice, but more or less surround the hair instead of projecting at an angle as do the ovoid lice nits. The small, sandy concretions are black or white in color and very hard, hence the name piedra—stone.

The stonelike concretions are caused frequently by the species *Trichosporum giganteum* and *Piedraia hortai*. If an infected hair is examined in sodium hydroxide or potassium hydroxide solutions, the nodule will be found to be made up of faceted cells matted to the side of or, at times, encircling the hair, but not invading it. In the case of *P. hortai* nodules, within the mass of cells are seen spherical to ovoid asci up to 30μ in diameter with eight elongate ascospores measuring approximately 40 to 45μ by 6 to 8μ, including long filiform appendages approximately 15 to 20μ long. *T. giganteum,* on the other hand, shows no asci. The fungi grow on Sabouraud's medium but better on carrot.

Piedra is fairly widespread throughout the tropical countries and in Europe, but may be found in the temperate zones. The disease was observed frequently in Colombia in women and the infection was thought to be due to the application of a mucilaginous preparation to their hair.

Manson-Bahr (1940) emphasizes that trichosporosis must not be confused with trichonocardiasis, which is a disease of the hair caused by *Actinomyces tenuis*. This infection is common on the axillary, scrotal, and facial hair in Europe and elsewhere. Also, it should not be confused with trichorrhexis nodosa, a nonparasitic

disease of the hair shaft, in which there appear irregular thickenings resembling nodes, the hairs often splitting at different points into brushlike bundles of fibers. It should also not be confused with moniliform hair, another nonparasitic condition which is congenital and hereditary.

Fungi Imperfecti

The *Fungi Imperfecti* include those organisms which do not produce sexual spores. In addition the asexual conidial stages of the *Ascomycetes* are included in this class. The classification of the *Fungi Imperfecti* is very unsatisfactory, owing probably to the fact that our knowledge is still very incomplete in regard to the life cycle of various fungi. The asexual spores, upon which the classification of the *Fungi Imperfecti* is based, are of two major types: thallospores and conidia. Thallospores are spores formed by the thallus or mycelium. Blastospores, chlamydospores, and arthrospores are examples of thallospores. A spore produced on a specialized hypha and freed by abstriction at the point of attachment is called a "conidium." The specialized hypha on which conidia are formed is called the "conidiophore." It must be kept in mind, however, that in many cases a resemblance between these conidia does not represent any true phylogenetic relationship. It has been found that in species heretofore grouped in the same genus, after the complete life cycle was observed, it became necessary to group these organisms in three different classes. The classification of the *Fungi Imperfecti* is, therefore, an artificial one not indicating true systematic relationships.

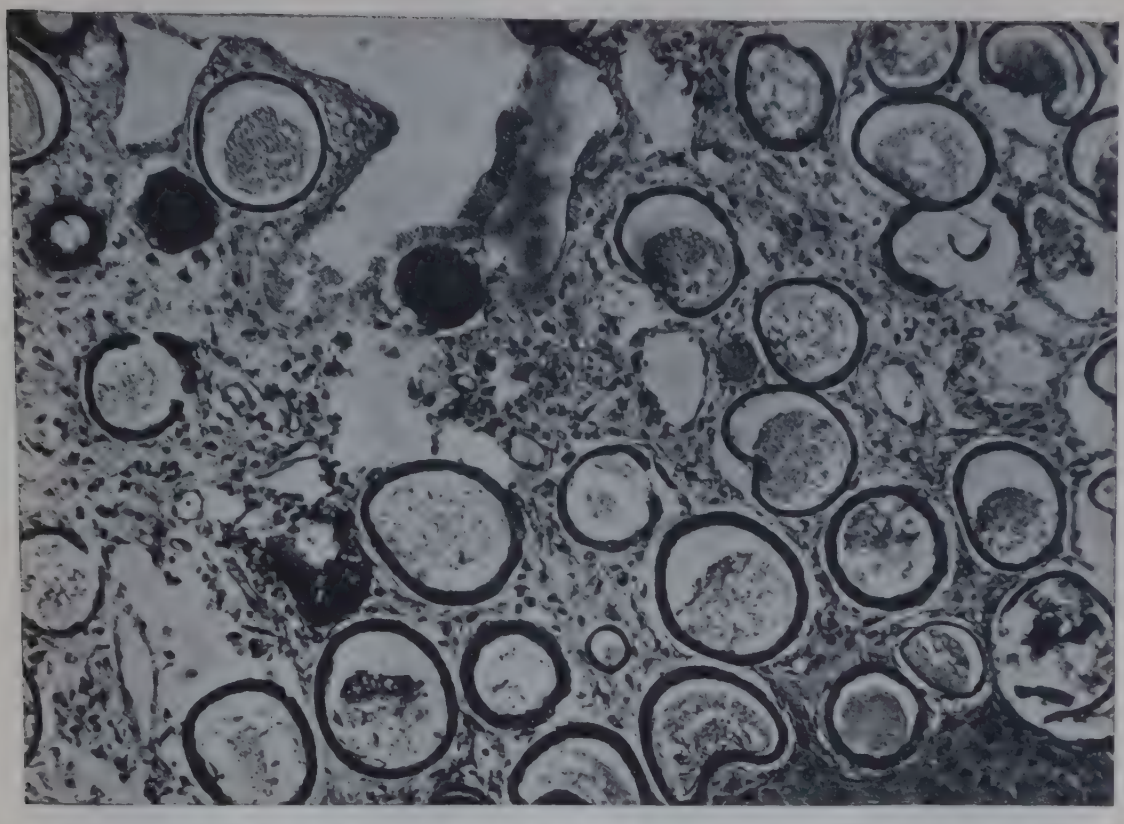
Members of the following genera will be discussed under affections due to the *Fungi Imperfecti:*

Sporotrichum	Paracoccidioides	Blastomyces	Rhinosporidium
Histoplasma	Candida (Monilia)	Cryptococcus	Aspergillus
Penicillium	Scopulariopsis	Malassezia	Microsporum
Trichophyton	Epidermophyton	Madurella	Indiella

Rhinosporidiosis. This is a polypoid infection, chiefly involving the mucous membranes of the nose, but rarely invading the ears, the lacrimal sac, the uvula, and more rarely, the mucosa of the penis. Dhayagude (1941) has described a case in a Hindu in which the parasite was found in nodules scattered over the body and there was no nasal or nasopharyngeal growth.

The disease is caused by a fungus, *Rhinosporidium seeberi,* which was first reported by Seeber in 1896 in Argentina. The organism was first considered a coccidial parasite, but the work of Ashworth (1922) indicated it is a fungus and not a protozoan. It is found within soft, very vascular, raspberry-like tumor masses, which are difficult to remove completely by reason of their friability and tendency to bleed profusely. The sporangia of the fungus measure 6 to 300μ in diameter; they have a thick wall and contain thousands of nucleated spores. Satisfactory cultivation of the parasite has not been reported and inoculation of animals has yielded negative results. The mode of transmission of the infection is unknown, but spores and ripe sporangia may be found in the nasal mucus.

The disease has been reported from India, Cochin China, Uruguay, Argentina,



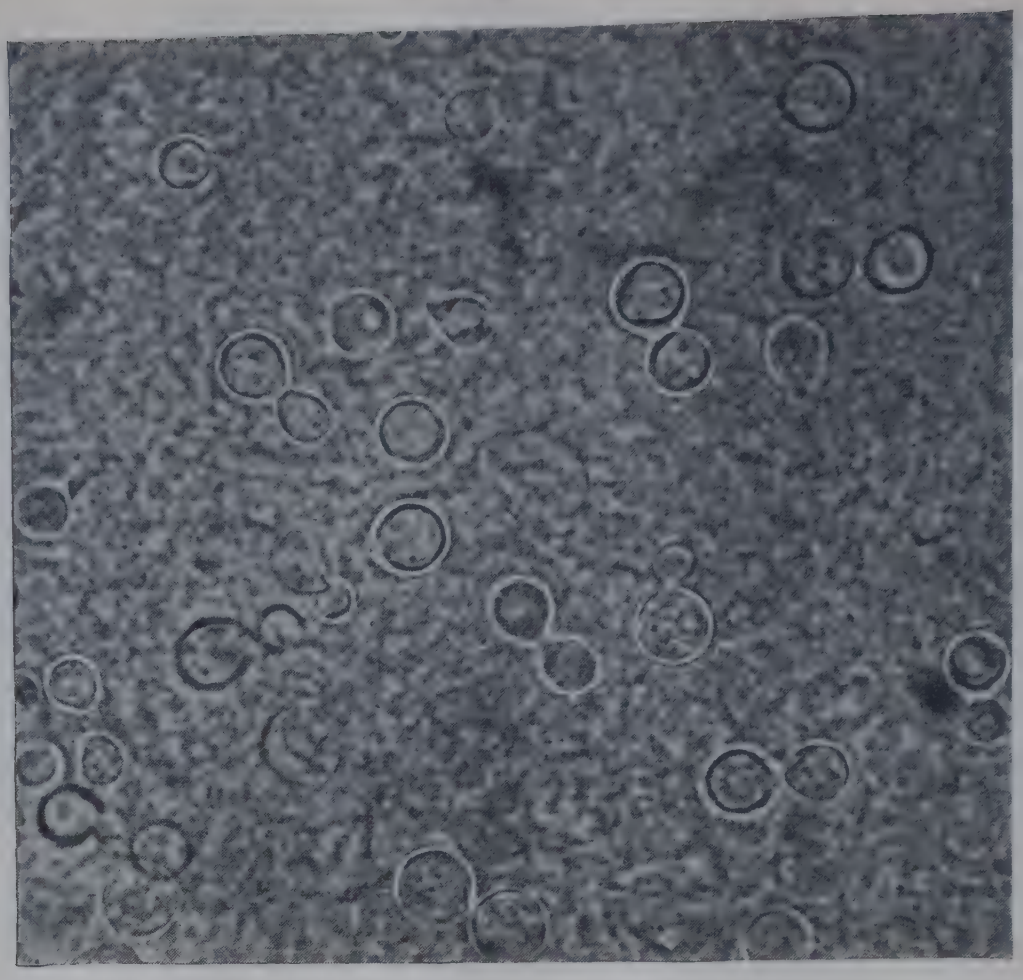
Rhinosporidium seeberi. Section from a nasal polyp. (U.S. Naval Medical School.)

and the United States. Apparently a closely related organism, *R. equi,* has been found in nasal cavities of the horse in South Africa.

The course of the disease is long and there is a marked tendency for it to recur.

North American Blastomycosis. Blastomycosis of the North American type, or Gilchrist's disease, is characterized by granulomatous and suppurative processes of the skin and subcutaneous tissues and sometimes of the lungs and other organs. Most of the cases first discovered in the United States were in the region of Chicago, but later they have been reported from many parts of the country. Marlin and Smith (1939) write that cases have been found in at least 28 states. The disease occurs more frequently in males than in females.

Gilchrist, in 1896, described the organism causing human blastomycetic dermatitis as encapsulated and budding in tissues with mycelial forms in cultures. The fungus is usually classed as *Blastomyces dermatitidis.* The organisms have been found in the pus from the lesions and sometimes in the sputum. In tissues, there are found spherical or ovoid cells singly or in groups, varying from 7 to 20μ in diameter. Budding forms are common, and the thick, highly refractile membrane makes the cells appear as doubly contoured. There is no mycelium in tissues. The infected material (pus, sputum, spinal fluid, etc.) should be cultured on blood agar incubated at 37° C., and on Sabouraud's agar incubated at room temperature. On blood agar the fungus grows slowly developing wrinkled, waxy colonies. Budding, yeastlike cells, identical with those seen in tissues, are present. Short mycelial fragments may also be noted. On Sabouraud's agar, mycelial development is enhanced. On about the third day small, creamy, prickled (coremia) colonies which show hyphae about 3μ in diameter appear. Numerous oval to round conidia are present attached to the hyphae near the septations. In older cultures,



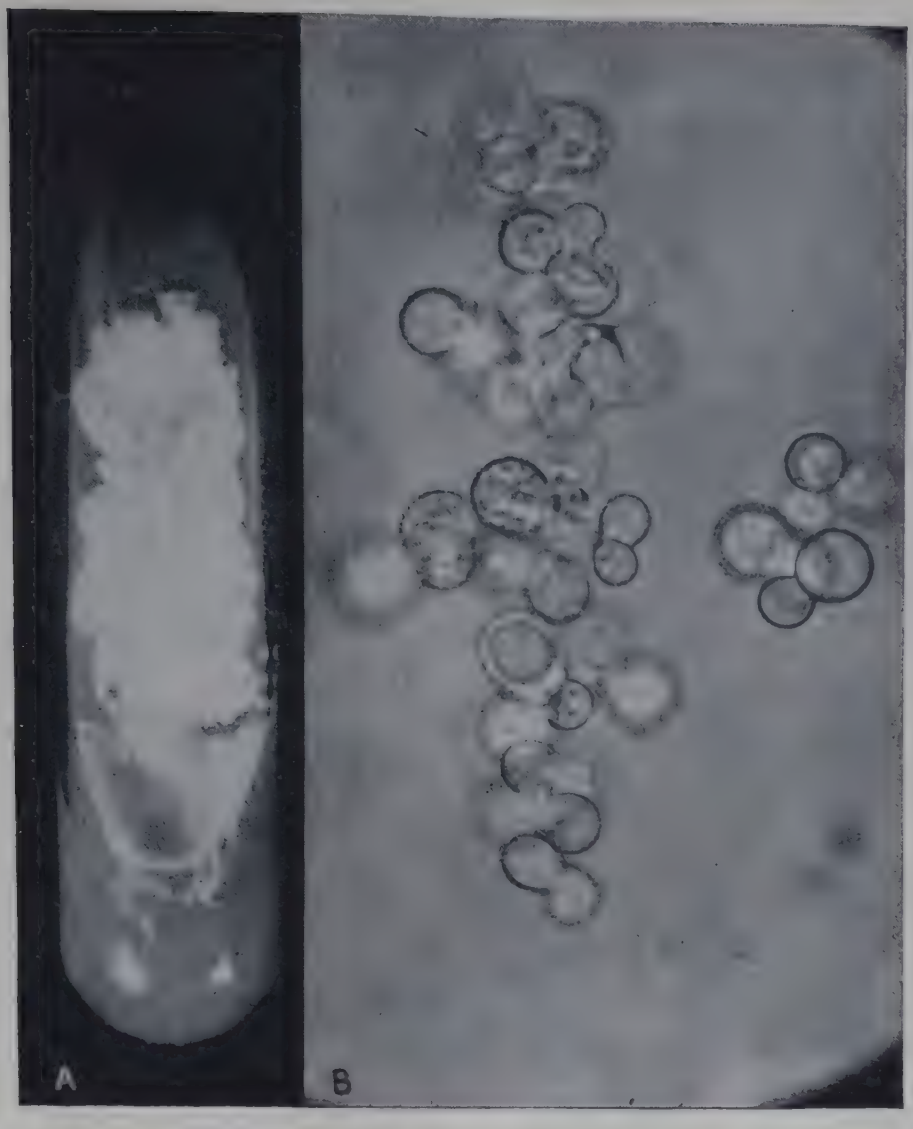
Blastomyces dermatitidis: round, double-contoured, budding, yeastlike cells in pus from subcutaneous abscess. (× 700.) (Courtesy, Conant et al.: Manual of Clinical Mycology, Philadelphia, W. B. Saunders Co.)

numerous chlamydospores are developed, 7 to 18μ in diameter, with unusually thickened outer walls. The mycelial phase can be converted to the yeastlike phase by subculturing to fresh media and incubating at 37° C.

The guinea pig is susceptible to inoculation, but the mouse is more so and may be inoculated either intraperitoneally or intratesticularly. The infection is fully developed in the laboratory animal by the third week, yeastlike bodies being present in the tissue nodules.

The disease may start as a skin lesion, or this may be secondary to a primary pulmonary invasion. The skin lesions may resemble a verrucose tuberculid, a syphilitic gumma, or sporotrichosis lesion. The exact diagnosis depends on laboratory examination. In generalized blastomycosis the lung is involved in more than 90 per cent of the cases, and in skin blastomycosis it is secondarily invaded in about one-third of such cases. In primary pulmonary blastomycosis a bronchopneumonia occurs from which the infection may generalize. There is less tendency to cavitation than in phthisis. The sputum is apt to be blood-stained and may contain the fungus. Next to the lungs and skin, the infection most frequently invades the kidneys, but the organism is not apt to be found in the urine unless there is invasion of the bladder or prostate. Bone and central nervous system involvement occur more rarely.

Martin and Smith (1939), who have analyzed the reports of more than 340



Blastomyces dermatitidis. (A) Yeastlike culture, 21 days, on beef infusion agar at 37° C. (B) Budding yeastlike cells from beef infusion glucose agar at 37° C. (× 700.) (Courtesy, Conant et al.: Manual of Clinical Mycology, Philadelphia, W. B. Saunders Co.)

cases including 13 of their own, recognize two clinical types of the disease: (1) a cutaneous one which proceeds as a chronic or subacute ulcerative process and which usually responds to treatment with iodides or radiation, and (2) a systemic form, a highly fatal disease, characterized by pulmonary infection and widespread distribution of lesions.

The sera of patients with extensive blastomycotic lesions will fix complement specifically with suspensions of *B. dermatitidis* or extracts of the organism. There is no cross reaction with other fungus infections, and the reaction is always negative in the absence of blastomycosis. No fixation of complement, however, occurs in undiluted sera of patients with the localized cutaneous form of the disease.

Most patients with blastomycosis respond positively to intracutaneous injections of the fungus or fungus products. The reaction is of the tuberculin type, resulting in maximal erythema in 24 to 48 hours, followed by the formation of sterile abscesses in very sensitive patients.

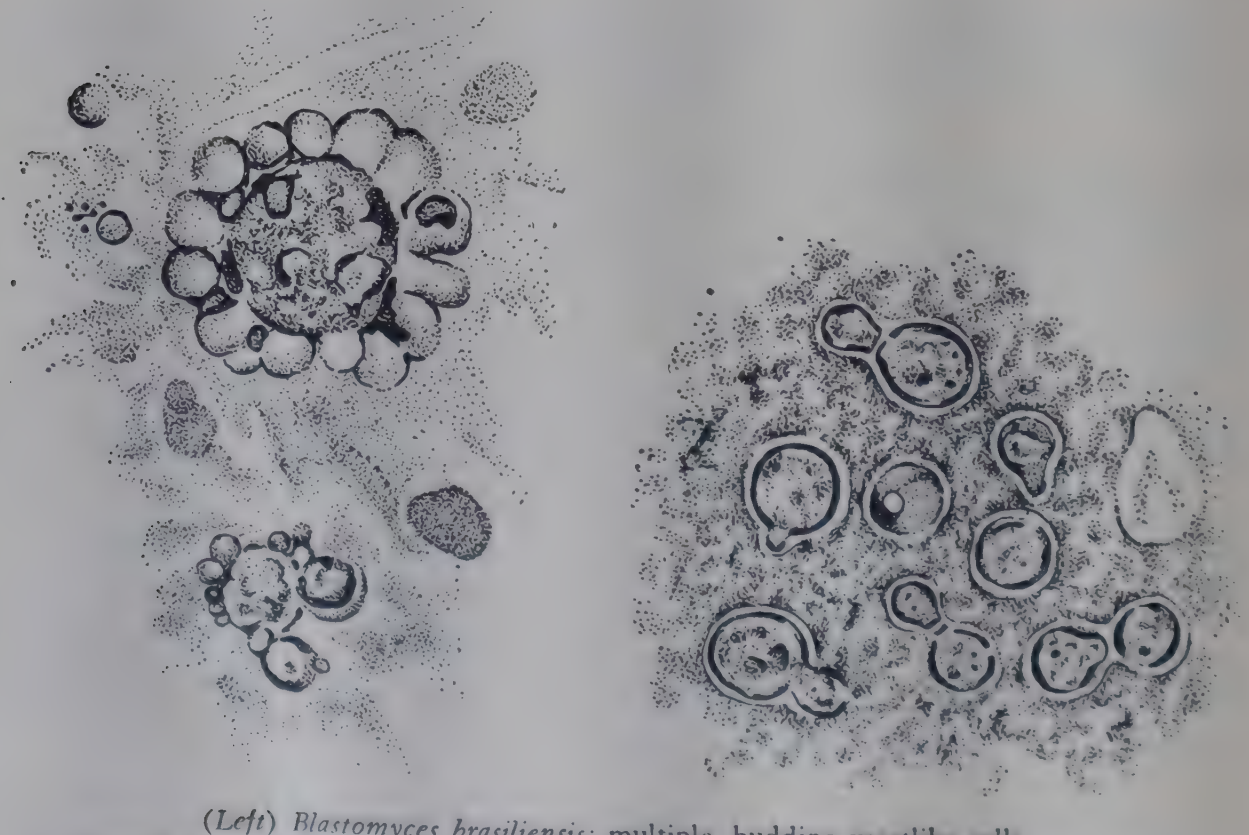
Dodge and Ayers isolated a fungus from the surface of the medulla in a case diagnosed as meningoencephalitis. They gave this parasite the name *Zymonema capsulatum*. Most

mycologists agree that the creation of a new species in this case is not valid since the organism is mycologically identical with *B. dermatitidis*.

South American Blastomycosis (*Paracoccidioidal Granuloma, Lutz-Splendore-De Almeida's Disease*). South American blastomycosis is a chronic granulomatous disease of the skin, mucous membranes, lymph nodes, and internal organs caused by *Blastomyces brasiliensis* (*Zymonema brasiliensis*). Most cases have been reported from Brazil with occasional cases from Argentina, Uruguay, Venezuela, and Peru.

A clinical feature typical of this disease is enlargement of the lymph nodes. Granulomatous and ulcerative lesions of the mucosa of the mouth and gastrointestinal tract separate South American blastomycosis clinically from North American blastomycosis. The infection is classified under the following clinical headings: (1) a cutaneous form characterized by cutaneous and mucosal lesions, particularly involving the mouth and nose; (2) a lymphangitic type beginning as localized lymph-node enlargement, most often of the neck, supraclavicular, or axillary regions; (3) a visceral form with lesions of the liver, spleen, pancreas, intestines, and other abdominal organs; and (4) a mixed type which involves skin and other organs to give a varied clinical picture.

Blastomyces brasiliensis is very closely related to the organism of Gilchrist's disease (North American blastomycosis). Both are yeastlike in tissues and in cultures at 37° C., and both develop a mycelium when cultured at room temperature. They are distinguished from each other by finding the single budding forms in the tissues from the North American type and the multiple budding forms from the South American type.



(*Left*) *Blastomyces brasiliensis*: multiple, budding yeastlike cells.
(*Right*) *Blastomyces dermatitidis*: round, double-contoured, budding yeastlike cells.

South American blastomycosis is sometimes confused with coccidioidomycosis. *B. brasiliensis* differs from *C. immitis* by a more restricted growth and spore formation on agar, and by reproduction by budding instead of endospore formation in tissues.

European Blastomycosis (*Cryptococcosis, Torulosis, Busse-Buschke's Disease*). Another ill-defined group of yeastlike fungi with pathogenic properties are those listed in the genus *Cryptococcus* (*Torula*), in which the organisms reproduce only by budding, do not produce mycelium or endospores, and do not ferment carbohydrates. They sometimes cause lesions of the skin, but appear to have definite affinities for the tissues of the central nervous system and the lungs, although they may produce destructive granulomatous lesions in other parts of the body. The respiratory tract is regarded as the probable portal of entry.

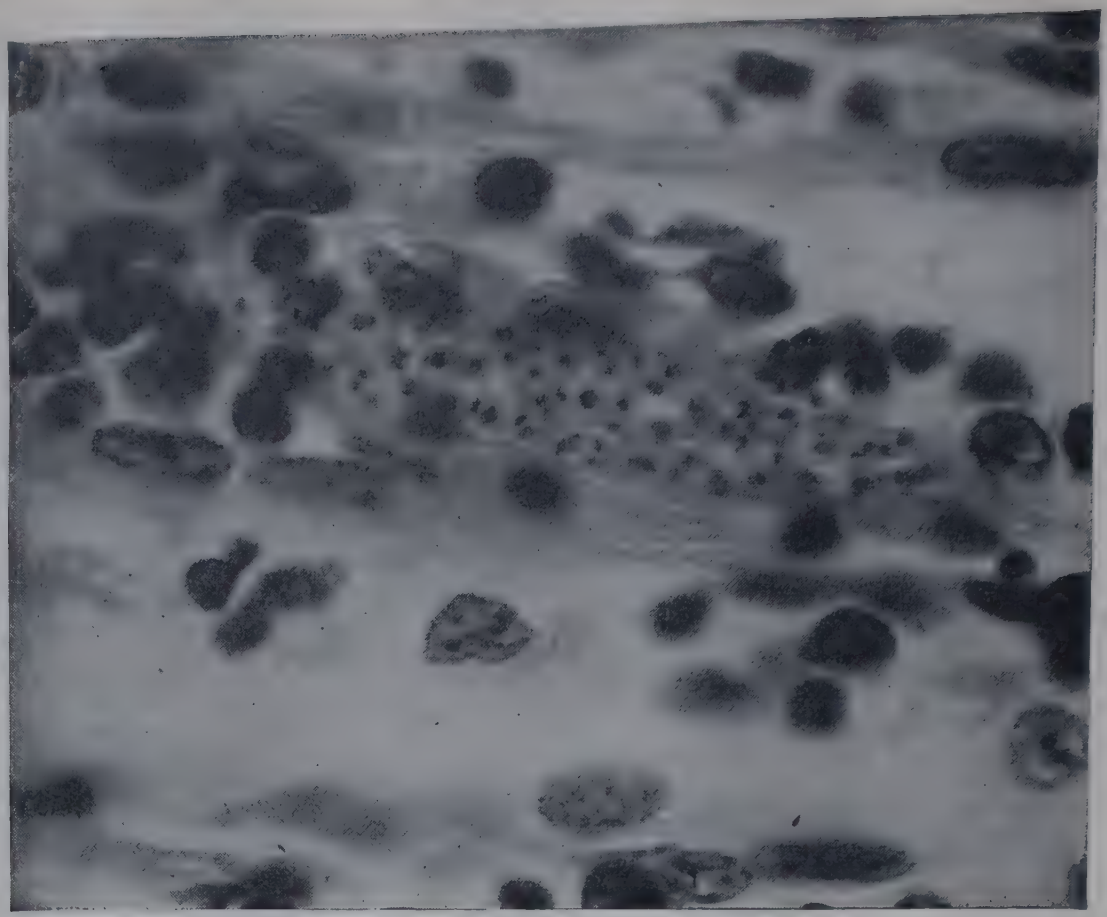
Freeman (1931) collected a number of cases with central nervous system involvement, which suggested neoplasm or encephalitis, but were associated with the presence of yeastlike organisms.

Although several synonyms (*Torula histolytica, Cryptococcus hominis*) are in common use, the correct name of the fungus is *Cryptococcus neoformans*. It reproduces only by budding, the yeastlike cells averaging 3 to 4μ in diameter. The whitish to yellowish colonies on glucose agar appear heaped up, smooth, pasty, shining, and thick. They do not cause fermentation, pellicle formation, or liquefaction of gelatin. In a case reported by Harrison (1928) from a cystic blastomycosis of the cerebral gray matter, the organism liquefied gelatin after 26 days.

The involvement of the cerebrum and the meninges in cryptococcosis suggests a brain tumor, particularly because of the eye symptoms, such as choked disc. A diagnosis may be possible by finding the yeastlike organisms in the cerebrospinal fluid. The prognosis is unfavorable.

Lymphangitis Epizootica. Lymphangitis epizootica has been recognized as a fungus infection, especially of the cutaneous lymphatics of the horse, since 1883, when Rivolta described *Cryptococcus farciminosus* as the cause of the disease. The lesions, which consist of nodules and ulcerations of the skin in situations rich in lymphatics, are frequently found where irritation from rubbing of the harness may result. The disease has been observed in Asia, southern and western Europe, northern Africa, Japan, and the Philippines. In 1906 a case of human infection with cutaneous lymphatic lesions of the chest wall, due apparently to the same organism, was observed in Manila and cultures of *Cryptococcus* were obtained. Brumpt (1936) mentions that instances of human infection may occur in individuals who care for infected animals, and Stovall and Almon (1941) say that the fungus has been inoculated into man. The parasite, however, appears to show no great virulence for human beings and there have been no reports of visceral involvement. Man is apparently only an accidental host.

Histoplasmosis. In 1906 Darling reported three fatal cases of a disease affecting the endothelial cells and quite similar to leishmaniasis clinically. The causative organisms packing the cells of the liver, spleen, adrenals, and bone marrow very closely resembled Leishman-Donovan bodies but did not show a blepharoplast.

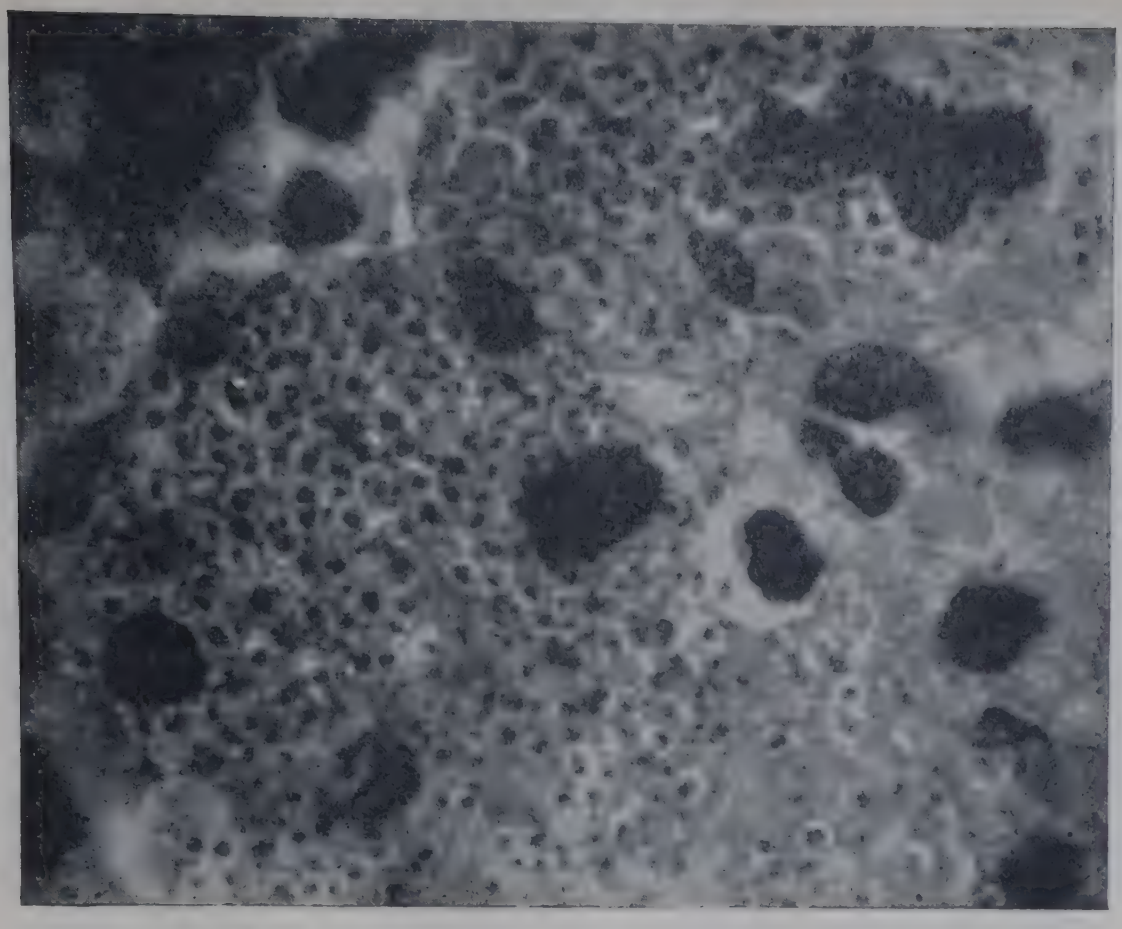


Leishmania in heart muscle. Note close resemblance to *Histoplasma capsulatum*. (See illustration on p. 245.)

Stitt has noted that in leishmaniasis a symbiotic relationship seems to exist between the protozoan and the infected cell, since very little cell damage seems to exist in stained sections. The organisms of histoplasmosis, however, appear to create much intracellular damage, rupturing the cell membrane, and scattering throughout the intercellular spaces.

Not until 1926 was another case of histoplasmosis reported—and from the United States. In 1932 the parasite was cultivated by DeMonbreun and recognized as a fungus, *Histoplasma capsulatum*. Cases are now being reported from other parts of the world from Austria, South Africa, Java, and the Philippines. Apparently *Histoplasma capsulatum* is the only pathogenic fungus found invading the reticulo-endothelial cells.

For a diagnosis, suspected material should be stained with Wright's or Giemsa's stain and examined for the presence of small (1 to 5μ) oval bodies in the mononuclear cells. Occasionally budding organisms can be demonstrated. Material should be inoculated onto blood agar to be incubated at 37° C. and onto Sabouraud's agar to be kept at room temperature. All cultures should be held at least a month before discarding them. On blood agar at 37° C. the growth becomes evident as moist, scattered, dull-white to tan colonies. Microscopically these colonies consist of small, ovoid, budding cells similar to those found in the tissues. Mixed with these cells are short fragments of mycelium. On Sabouraud's agar at room temperature the fungus grows slowly, producing a white, cottony, aerial mycelium which later turns buff to brown in color. Microscopically these colonies show



Histoplasma capsulatum in macrophages of liver.

branching, septate hyphae bearing small, round to pyriform, smooth spores (2 to 3μ in diameter) on short, lateral branches or sessile on the sides of the hyphae. At this stage of growth the culture may be mistaken for *B. dermatitidis,* but soon the typical round to pyriform, tuberculate chlamydospores are produced (7.5 to 15μ in diameter) and the mycologic diagnosis may be established.

The parasite has been found in dogs but never as a saprophyte. The infection has been transmitted to young dogs, both parenterally and by mouth, both with material from an infected dog and by inoculation of cultures. Experimental infections have also been reported in monkeys and mice. Hansmann and Schenken (1933) have reported a case in man and described experimental infection in guinea pigs, rabbits, dogs, and rats.

Palmer (1905) reported that there was a high correlation between the occurrence of pulmonary calcification and sensitivity to histoplasmin. Skin reactions to histoplasmin were noted in 61 per cent of student nurses in Kansas City showing calcification and 11.8 per cent for nurses from Philadelphia.* Emmons, Olson, and Eldridge (1945) showed that the histoplasmin Palmer used was not specific for histoplasmosis but induced skin reactions in guinea pigs infected experimentally with either *Histoplasma, Blastomyces, Coccidioides,* or *Haplosporangium.*

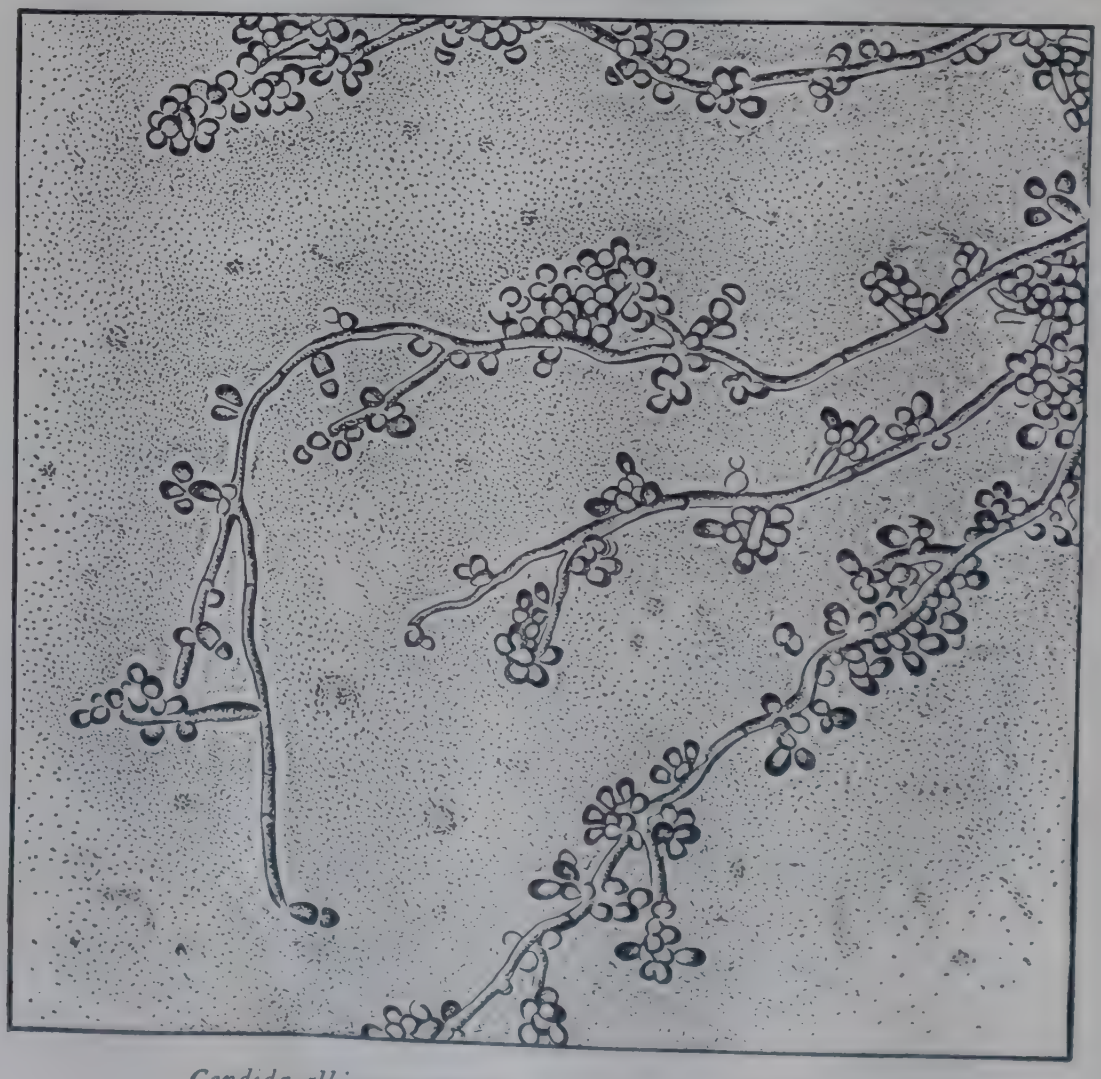
The prognosis of histoplasmosis is generally unfavorable when the fungus has become widely disseminated. Only a few instances of recovery have occurred.

*Palmer reported that of 2141 nurses in whom reactions to both tuberculin and histoplasmin were negative only about 1 per cent showed pulmonary calcification.

Candidiases (*Moniliases*). There is a large group of fungus infections which have been attributed to various yeastlike organisms, many of which show mycelial development in cultures. The best known of these candidiases is "thrush," in which grayish-white membranous patches form on the mucous membrane of the gums, tongue, buccal cavity, and pharynx, chiefly affecting marasmic infants or feeble old people. The fungus rarely attacks well-nourished individuals.

Species of *Candida* (*Monilia*) are simple yeastlike fungi which reproduce by budding, form a septate mycelium under suitable conditions, and do not show the formation of ascospores. They differ from the yeasts by the formation of mycelium, and cannot be classed with the *Ascomycetes* since ascospore formation has not as yet been observed.

The infections caused by the genus *Candida,* in contrast to blastomycosis caused by *B. dermatitidis,* are characterized by their generally superficial nature and by the rather feeble invasive powers of the organism. It is difficult to distinguish between the *Candidae* of the gastrointestinal tract in health and disease, since *C. albicans* is present in over 50 per cent of gastric samples and 10 per cent of feces in healthy individuals. Todd (1937) has found *C. albicans* in the mouth or throat of 14 per cent of 1000 healthy persons.



Candida albicans: septate mycelium with blastospores.

The most common of the *Candidae* producing lesions in man is *C. albicans* (*Oidium albicans, Monilia albicans*). It is the cause of thrush and may appear in lesions in both the mycelioid and budding stages, the latter predominating. The mycelium has numerous budding cells and, on cornmeal agar, large terminal chlamydospores.

The common pathogenic species, *C. albicans,* can usually be identified solely on the basis of examination of cornmeal agar plate culture. The inoculum is placed in three or four parallel lines across the agar plate, the needle cutting through the agar to force some of the inoculum below the surface. After incubation at room temperature for four or five days, most strains of *C. albicans* form hyphae extending laterally from the streak. The hyphae bear characteristic clusters of buds and thick-walled, spherical chlamydospores.

The culture on dextrose agar is creamy white and has a pasty consistency. It has a yeastlike odor and consists of round or oval budding cells. Anaerobic conditions and the absence of fermentable carbohydrates increase mycelium formation.

It is astonishing how many species are described in the literature based on a single cutaneous, pharyngeal, or pulmonary condition from which yeastlike bodies were isolated. Competent mycologists have in recent years stressed cultural



Candida albicans: chlamydospores.

studies with various media as is required for bacteria, along with determination of pathogenicity. (See Table 14.)

Besides thrush and perlèche (mycosis of the angle of the mouth, characterized by macerated whitish patches) there are candidiases of the perineal or inguinal regions of infants, particularly those poorly nourished. Probably many of the *Candidae* are saprophytic and only develop in individuals of lowered resistance. Some of the so-called body eczemas and dysidroses of hands and feet are associated with candidiasis. Many cases of athlete's foot, particularly where the toes are closely proximated, belong to the candidiases, although by far the greater number of such cases belong to the ringworm group. Vaginal discharges may also be due to infection by species of *Candida,* as well as to *Trichomonas vaginalis.* Generalized candidiasis, which is rare, manifests itself by widespread cutaneous, circumscribed lesions. Desquamation, following pustulation and maceration, exposes a bright-red, moist surface, with a raised, thickened periphery. Candidiasis of considerable duration has a peculiar yeasty odor. Bronchial or pulmonary candidiasis is said to occur. The lesions spread from the oral cavity to the bronchi and the lungs. All stages of the disease may progressively be seen—a mild type with slight cough, with greenish or yellowish, mucopurulent sputum, but no fever, to the picture of pulmonary tuberculosis with irregular fever, night sweats, weight loss, frequent coughs, and expectoration of a tuberculous nature. In the later stages the disease is frequently fatal.

Pityriasis Versicolor (*Tinea Versicolor*). This very common skin infection is characterized by dirty yellowish-brown spots occurring upon covered parts of the body, especially under the clavicles. These spots are sometimes referred to as "liver spots." The plaques are not elevated and do not show inflammation. A vigorous sweep of the thumbnail (a sterile instrument is preferable) superficially across the patch does not bring blood (*signe du coup d'ongle*). The scrapings provide material showing an abundance of mycelium and spores of *Malassezia furfur.* The hyphae are from 2 to 3μ in diameter and the spores from 3 to 8μ. The spores are very refractile and may show budding. Most observers have been unable to cultivate the fungus. Since the diagnosis can usually be made by direct examination of skin scrapings, cultural methods have been largely abandoned.

Erythrasma. This disease is sometimes confused with pityriasis versicolor. Erythrasma is a distinct skin infection caused by *Actinomyces minutissimus* (*Microsporum minutissimum*). The mycelium averages 1μ in diameter, often fragmented into bacillary forms. Cultures are usually negative. Erythrasma spots are dark red to brown (usually occurring in the region of the groin or axilla).

Dermatophytoses. Five genera of dermatophytes were recognized by Sabouraud: *Microsporum, Trichophyton, Endodermophyton, Epidermophyton,* and *Achorion.* The genera *Achorion* and *Endodermophyton* are, however, mycologically indistinguishable from members of the genus *Trichophyton.* All of these invade the skin and hairs. The genus *Trichophyton* was further subdivided into subgenera on the basis of the position of the fungus within the hairs: *endothrix Trichophytons,* growing entirely within the hairs; *ectothrix Trichophytons,* growing mainly

Table 14

DIFFERENTIATION OF CANDIDAE

Species	*Colony on Blood Agar*	*Mycelial Growth on Cornmeal Agar*	*Agglutination with C. Albicans Antiserum*	*Pathogenicity for Rabbits*	*Sugar Fermentation*			
					Dextrose	*Sucrose*	*Lactose*	*Maltose*
C. albicans	Dull grayish white	Treelike, chlamydospores on tips of branches. Spherical spore clusters. Buds usually at ends of mycelial segments	+	+	AG	A	—	AG
M. parapsilosis (*C. parakrusei*)	Pearly white. Size 0.7 mm. Smooth and circular	Produced with difficulty. No chlamydospores. Irregular spore clusters. Buds usually at ends of mycelial segments	±	—	AG	—	—	—
C. tropicalis	Grayish white. Size 2 mm. Mycelial fringe	Mycelium abundant. No chlamydospores. Buds anywhere on mycelium	+	Temporary skin lesions in large doses	AG	AG	—	AG
C. krusei	Dull grayish white. Size 0.2 to 1.0 mm. Variable in shape	Naked threads with branching at wide intervals. No chlamydospores. Buds often in whorl at tips of mycelium	—	—	A	—	—	—
C. pseudotropicalis	Size 0.5 mm. Variable in shape	Similar to *C. parakrusei*	—	—	A	A	A	—
C. stellatoides	Elevated central zone with radiating "arms"	Similar to *C. albicans*	—	—	A	—	—	A

Adapted from a Practical Classification of the Candida by Martin, Jones, Yao, and Lee (1937).

on the surface of the hairs; and *neo-endothrix Trichophytons*, growing mainly within the hairs but occasionally showing growth on the surface of the hairs.

Key to the Dermatophytes

(From Henrici)

Lesions of the Scalp

I. Yellow crusts or scutula present. Clinical favus.

A. Hairs tend to split longitudinally. Colonies waxy, wrinkled, little or no aerial mycelium. Hyphae coarse, distorted, tips swollen and branched. No macroconidia, few conidia.

Trichophyton schoenleini

B. Colonies white, downy. Conidia and macroconidia formed.

Microsporum quinkeanum

C. Colonies yellowish-brown with abundant aerial mycelium and very numerous macroconidia.

Microsporum gypseum

D. Colonies glabrous, wrinkled, reddish-violet.

Trichophyton violaceum

II. No scutula present. Hairs tend to break off square.

A. Suppurative reactions (as follicular abscesses, pustules, or kerion) absent (except with species of animal origin).

1. Hairs broken off at a uniform height, several millimeters above the skin. Much scaling of the epidermis, highly contagious in most cases. Spores on outside of hairs, angular, forming a mosaic. In cultures many spindle-shaped macroconidia with thick, usually rough walls, multicellular (except *M. audouini*).

MICROSPORUM

2. Hairs mostly broken off flush with the skin, leaving black points. Little scaling of the epidermis. Not so contagious. In cultures, conidia variable in size, macroconidia few or lacking.

a. Grows entirely within the hair, both mycelium and spores.

Endothrix TRICHOPHYTON

b. Grows mainly within the hair, but a few hyphae and spores can be found on the exterior.

Neo-endothrix TRICHOPHYTON

B. Suppurative reactions occur. Lesions of the smooth skin also frequently present. Some strains of animal origin. Fungus grows in and on the hair, spores mostly external, arranged in rows, not in mosaic.

Ectothrix TRICHOPHYTON

1. Spores in hair—5 to 8μ in diameter.

Section Megaspores

2. Spores in hair—3 to 4μ in diameter.

Section Microides

3. Fusiform macroconidia present in culture.

MICROSPORUM (species of animal origin)

Lesions of the Smooth Skin

I. Eczema-like lesions confined to moist parts, as inner surfaces of thighs, axillary regions, between fingers and toes, soles of feet. Not found within hairs. In culture, greenish-yellow, no conidia, macroconidia egg-shaped to clavate, thick, smooth walls.

EPIDERMOPHYTON (*E. floccosum*)

II. Lesions not as above, generally involving hands, arms, general body surface, face, neck.

A. Lesions form intricate patterns of concentric rings with marked scaling. Hairs not invaded.

Trichophyton concentricum

B. Lesions are reddish patches, not raised above the skin level, round to irregular in form, darker at the border, forming rings. Tending to heal in center, new attacks may occur, forming concentric rings.

Generally MICROSPORUM
Sometimes Endothrix TRICHOPHYTON

C. Lesions are elevated plaques, reddish, round, or oval; scaly. Pustules frequently present at the border.

Ectothrix TRICHOPHYTON

Microsporosis. Microsporosis is a disease of the scalp and smooth skin of children. Species of *Microsporum* of animal origin cause infection in both adults and children. Microsporosis is sometimes referred to as ringworm, tinea, or tinea circinata. It is customary to recognize a human type of microsporosis, caused by *Microsporum audouini,* and an animal type, caused by *Microsporum canis* (from the dog). The human type is important only for the scalp, as the lesions which may appear on the glabrous skin are rather insignificant and come from scalp transfer. It does not affect the beard. The areas of alopecia of the human type show grayish scales, have definitely outlined borders, and are covered with diseased hairs broken off about 3 to 5 mm. above the scalp surface. The affected areas never contain any normal hairs. On epilating one of these grayish stumps of hairs there is evident a whitish collar of polyhedric spores (2 to 3μ) on the surface and hyphae growing within the shaft of the hair. This infection attacks young children almost exclusively, and Brumpt advises considering an infection of animal origin when one of these small-spored ringworms is found in an adult. The human type usually causes little pustulation, although secondary bacterial infection due to scratching may occur. Castellani (1934) reported that in Ceylon and China a species, *M. scolaceous* (Bodin), leads to permanent alopecia.

The animal species of dog origin, *Microsporum canis* (*M. lanosum*), produces both tinea tonsurans and circinate herpes in children and adults. The lesions are generally dry and scaly. The cultures grow more rapidly than those of the human type and they show more abundant fuseaux.

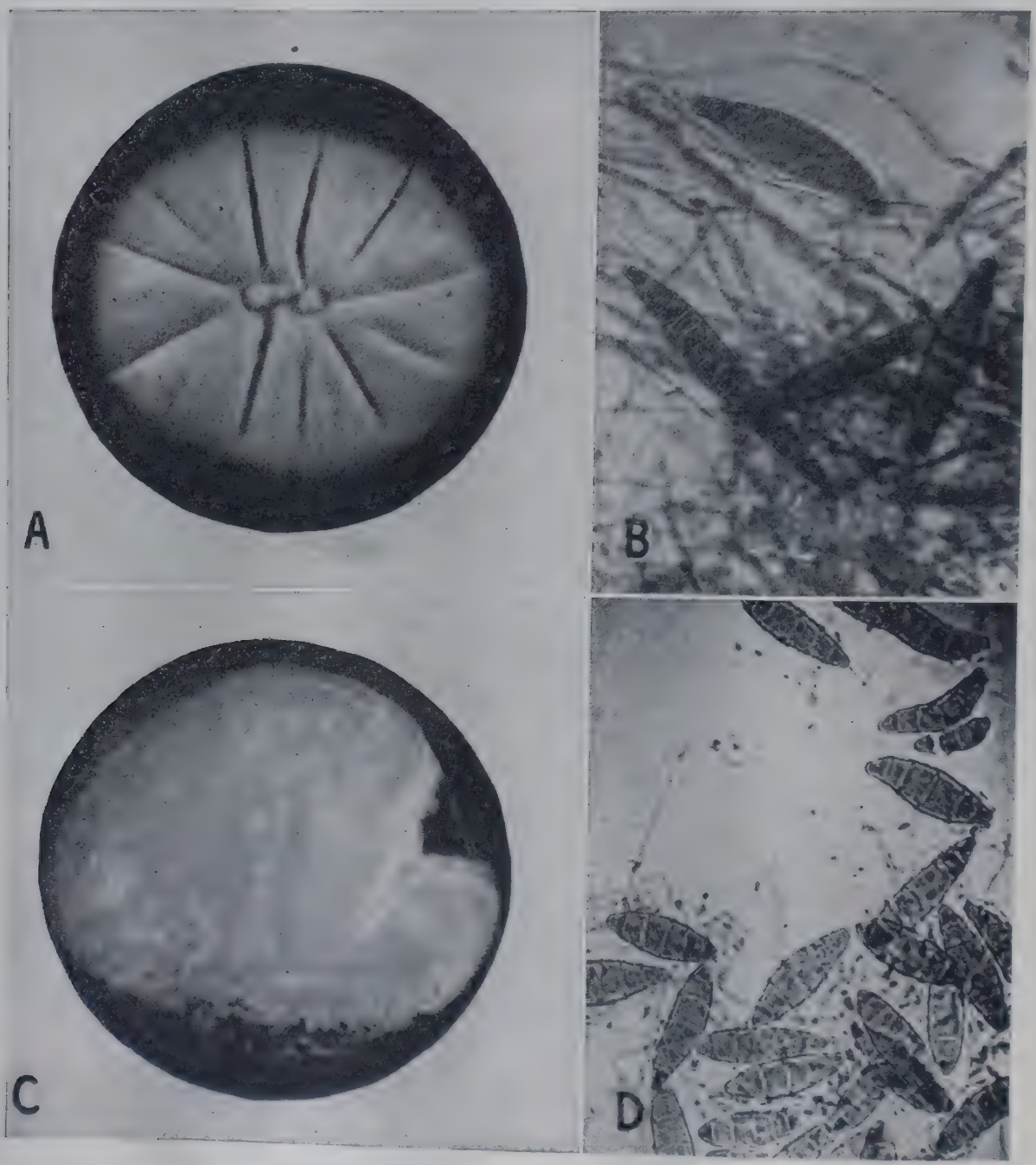
The species causing ringworm in the cat is often referred to as *Microsporum felinum,* but it is probably the same as *M. canis.* It seems to prefer the glabrous skin, producing dry, erythematous lesions which may go on to pustulation. It grows very rapidly in cultures. Some kerions are due to the cat ringworm.

Microsporum audouini and *M. canis* may be differentiated by the characteristics tabulated below (adapted from Plaut and Grutz, 1927):

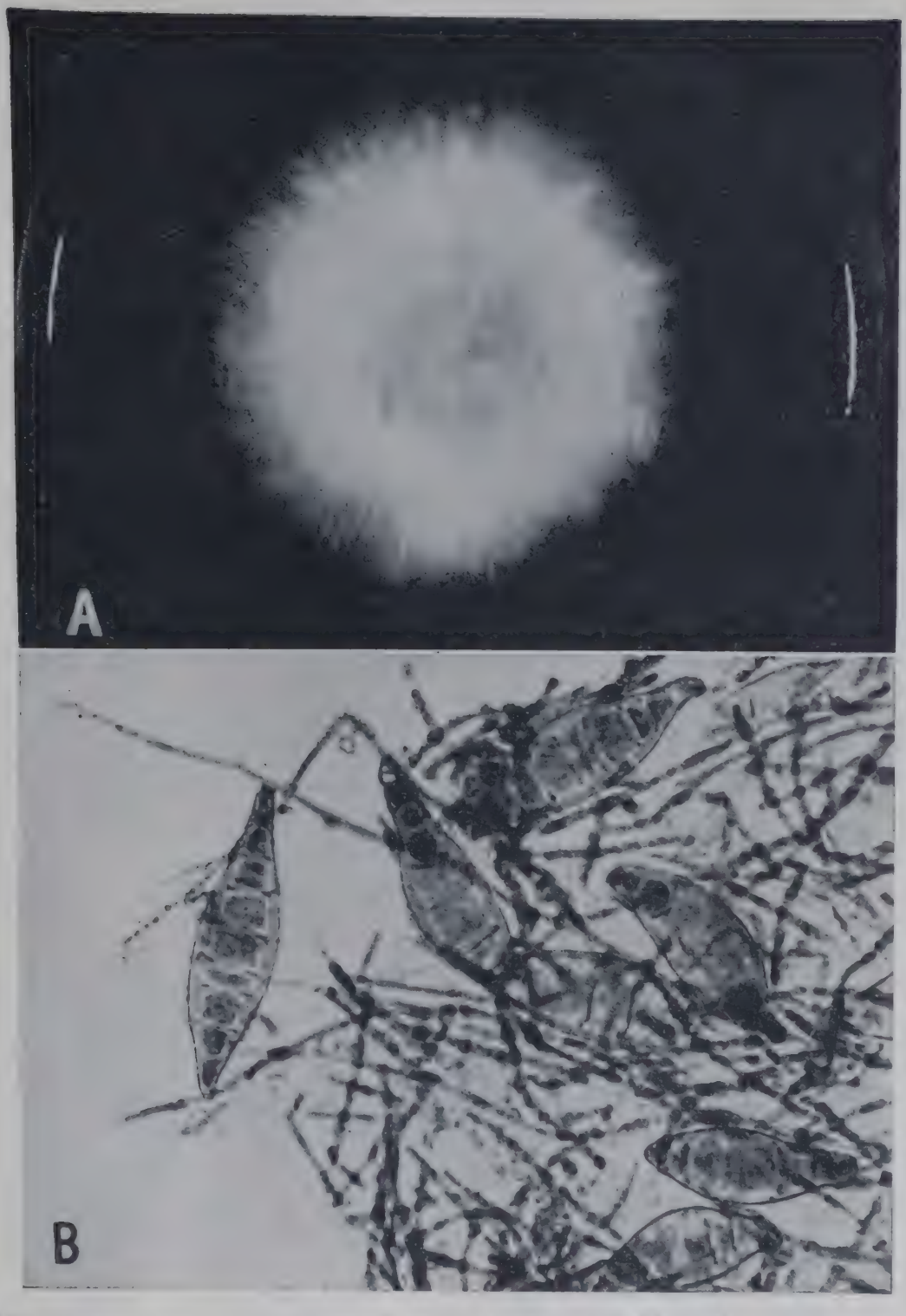
M. Audouini	*M. Canis*
1. Highly contagious, causing epidemics in schools.	1. Less contagious, may cause epidemics in families.
2. Of long duration, resistant to treatment.	2. Of shorter duration, about one year.
3. Only the head involved, exceptionally areas in the immediate vicinity.	3. Frequently skin lesions occur at a distance from the head.

M. Audouini	*M. Canis*
4. Inflammatory reactions mostly lacking if untreated.	4. Inflammatory reaction present without any irritation from treatment.
5. Cultures grow slowly.	5. Cultures grow more rapidly, and colonies attain a larger size.
6. Colonies remain snow-white.	6. Colonies become reddish in center when spindle spores are formed.
7. Only rarely transmissible to animals from cultures.	7. Rabbits and guinea pigs easily infected from cultures.

Cultures of *Microsporum* are characterized by cottony, matted, or powdery aerial mycelium varying in color from white, buff, to deeper shades of brown.



(A) *Microsporum audouini* on Sabouraud's glucose agar. (B) *M. audouini:* multiseptate, spindle-shaped macroconidia. (× 250.) (C) *M. gypseum* on Sabouraud's glucose agar. (D) *M. gypseum:* multiseptate, ellipsoid macroconidia. (× 250.) (Courtesy, Conant et al.: Manual of Clinical Mycology, Philadelphia, W. B. Saunders Co.)



(A) *Microsporum canis* on Sabouraud's glucose agar. (B) *M. canis:* multiseptate, spindle-shaped macroconidia. (× 480.) (Courtesy, Conant et al.: Manual of Clinical Mycology, Philadelphia, W. B. Saunders Co.)

Microscopically the genus can be immediately identified by the large (8 to 15μ in width, 40 to 150μ in length), rough, thick-walled, spindle-shaped macroconidia (fuseaux). Small (3 to 5μ) microconidia are borne along the sides of the hyphae. Racquet hyphae, pectinate hyphae, and chlamydospores are often found.

M. AUDOUINI. This fungus is slow growing. The colony consists of a velvety mycelium which remains white. The reverse of the colony is reddish brown to orange in color. A few large, multiseptate fuseaux are seen in the early stages of colonial growth. These fuseaux, typical of the genus, are never numerous and soon disappear. Microconidia are

present, borne laterally along the hyphae. Racquet mycelium, pectinate bodies, and chlamydospores are found.

M. CANIS. The colony develops rapidly with a buff or light brown, cottony aerial mycelium. Colonies become reddish in the center when spindle spores are formed. The reverse of the colony is reddish-brown to orange in color. Numerous large, multiseptate, spindle-shaped macroconidia (fuseaux) are present. Racquet hyphae, pectinate bodies, chlamydospores, and sometimes spiral hyphae are found.

TRICHOPHYTOSIS. Species of the genus *Trichophyton,* like those of the genus *Microsporum,* produce ringworm of the scalp, glabrous skin and beard, but in addition the nails, inguinal region, toes, interdigital spaces, axillae, and the body in general may be affected. The lesions vary according to the site and species responsible. Depending on the position of the fungus within the hair, it is customary to divide members of the genus *Trichophyton* into several groups.

ENDOTHRIX TRICHOPHYTON AND NEO-ENDOTHRIX TRICHOPHYTON GROUPS. In the endothrix group, the hyphae are confined to the interior of the hair, whereas in the neo-endothrix group, in addition to hyphae in the interior, there are a few hyphae growing along the outside of the hair shaft. Three subtypes have been described in the endothrix group of Trichophyton infections, *Trichophyton tonsurans, T. violaceum,* and *T. sabouraudi* infections.

In the first type there are many normal hairs in the region of alopecia. The scales covering the affected spot, if gently removed, show flattened, diseased hairs lying beneath them, often assuming bizarre shapes. This infection rarely itches. It is very contagious. This ringworm may also occur on the face, neck, and hands of children showing the infection as red areas with little vesicles or papules. The nails may be invaded.

The *T. sabouraudi* subtype is quite common in western Europe. In this infection the hairs break off close to the scaly scalp patches, and show as black dots on the scalp surface, the "black dot" ringworm. This fungus may cause trichophytosis of the hands, and secondarily of the nails. The beard may be invaded.

Clinically the lesions produced by the *neo-endothrix Trichophyta* are similar to those produced by the endothrix group. Differentiation is made by determining the relative position of the hyphae to the hairs. The type species of the neo-endothrix group is *Trichophyton flavum.*

ECTOTHRIX GROUP. Members of the genus *Trichophyton* are placed in this group when the mycelium is found both within the hair shaft and in the root sheath surrounding the hair. This has two main subgroups, the microides, or small-spored type, and the megalospore, or large-spored type.

Among the large-spored members of this group *Trichophyton megnini* (*T. roseum*) is said to be rather common in the north of England, where its spread seems to be through the barber shops, rather than from contact with animals. It has spread rather widely since World War I, being reported from France, Germany, and the United States, particularly Philadelphia. The lesions involve the skin or hair follicles, but chiefly the beard, and are rather dry, showing no tendency to suppuration.

In the microides group, the common fungus is *Trichophyton mentagrophytes*

(*T. gypseum, T. interdigitale, T. pedis*). In sycosis vulgaris, "barbers itch," the lesions consist of a folliculitis of the scalp or regions of the beard complicated, in addition, by infection with pyogenic cocci. The inflammation results in a suppuration of the follicles containing the dead hairs. The same fungus when it invades the tissues more deeply is probably the most common cause of "kerion," in which there are patches riddled with openings from which pus oozes on pressure. These patches are often on the backs of the hands and on the forearms in addition to the scalp, and occur frequently in those who work with horses. When on the face or scalp the affected hairs can be easily and painlessly extracted from the root sheath; they are, however, quite brittle and may break off. On mounting in a clearing solution (potassium hydroxide solution) strings of spores (5μ) may be found on the hairs extracted from the periphery of the lesion or in the pus. The sheath at the base of the hair has spores varying greatly in size (2 to 11μ). The microides spores of the sheath may be confused with the spore sheath of *Microsporum audouini,* but the former are arranged in chains, whereas the latter show masses of polyhedric spores, about 3μ in diameter.

Grossly, colonies of the genus *Trichophyton* may appear cottony, granular, or powdery, velvety or smooth and waxy depending on the species. Pigmentation is variable, and colonies may appear white, pink, red, purple, brown, yellow, or orange. Subcultures of the colonies may result in complete or partial loss of pigment. The characteristics of the colony serve to divide the genus into the gypseum, niveum, rosaceum, and faviform groups.

Microconidia are numerous and are typically small, thin-walled, single-celled, clavate conidia, 2 to 4μ, and are borne in grapelike clusters (en grappe) or singly from the sides of the hyphae.

Macroconidia (fuseaux) may or may not be present. When present, they appear as large, multicelled, smooth, thin-walled, clavate conidia (4 to 6μ in width and 10 to 50μ in length). Racquet mycelium, chlamydospores, and spiral hyphae are sometimes observed.

Dermatophytids. In recent years a number of dermatologists have reported the



Macroconidia. (*Left*) *Microsporum canis.* (*Center*) *Trichophyton sp.* (*Right*) *Epidermophyton floccosum.*

appearance of lesions of the skin resulting from hypersensitivity to certain fungi and have termed such lesions "ids." Allergy is said to be the *sine qua non* of their existence. The term "trichophytid" has been sometimes used synonymously with dermatophytid but the typical skin eruption may be due to *Microsporum* as well as *Trichophyton* infections. Sutton (1939) points out that in a dermatophytid due to *Trichophyton* infection the trichophytin reaction becomes positive.

The dermatophytids are fungus-free lesions. When they occur they are regarded as the result of hypersensitivity of the skin to fungus products. Thus, it is believed that fungus-free lesions of the hands may be an allergic response and occur as the result of fungus infection of the feet.

Lewis and Hopper (1943) emphasize that certain criteria must be met before the diagnosis of dermatophytid can be made. A primary focus of infection in which fungi can be demonstrated must be present. Fungi are not present in the dermatophytid. The reaction to the intracutaneous test with trichophytin should be positive. The eruption disappears spontaneously when the primary focus of infection is eradicated.



Tinea pedis.

A positive trichophytin reaction, however, does not necessarily indicate *Trichophyton* infection, since it has been impossible to demonstrate the fungus in many positive reactors. Lewis, Stovall, and Almon believe it may be of value in a negative way in excluding *Trichophyton* infection.

Favus. This disease usually affects the hairs of the scalp, although sometimes the glabrous skin and nails are invaded. It is characterized by golden-yellow, cup-shaped crusts (scutula), which form about the hair-follicle orifices. The scutulum is made up of tangled mycelium surrounding the hair with vertical mycelium externally and, underneath, a pus-cell layer. The scutula may remain isolated, or



Dermatophytid of hands.

form dirty crusts on coalescence. If this impetiginous crust is removed the yellow color appears. The odor of old favus lesions is that of a mouse nest. The causative fungus is commonly *Trichophyton schoenleini*. In addition, *T. violaceum*, *M. gypseum*, and *T. quinkeanum* have been isolated from favus lesions. The evolution of the condition is slow. Some European dermatologists believe the disease is contracted only during infancy but that once it is acquired it never disappears entirely spontaneously. However, Brumpt reports that there are exceptions to this latter view. Sutton states infection is possible in an individual of any age. The disease is moderately infectious and spreads by direct contact.

The affected hairs are lusterless and grayish, and although more fragile than normal hair, can be epilated entirely. Scarring may result. The favic hair is filled with air bubbles. The septate hyphal chains vary greatly in diameter (2 to 5μ). Cultures on Sabouraud's medium are like yellow beeswax and resemble cerebral convolutions. The growth is very slow and on microscopic examination there are very few spores. On the glabrous skin, scutula may be found, but scaly patches are more common.

Involvement of the nails is rare and always follows lesions elsewhere. The toenails are very rarely attacked. In the absence of a prior case in a human being one must suspect infection from an animal source, particularly from mice, caused by *T. muris*. *T. muris* produces a serious favic disease in mice, attacking the head and leading to blindness. In the human infection the lesions are common on the body (like herpes circinatus) and also on the head.

Favus due to *T. muris* is reported as common in Germany, less so in England and France. The classical favus is found in Japan, England, and America. In the United States, cases are more common in immigrants from eastern Europe. The disease is also common in Mexico.

Epidermatophytosis. *Epidermophyton* may be separated from other genera causing dermatophytoses by the fact that its members do not attack the hairs. In *Epidermophyton* infections the fungus is found characteristically in the stratum corneum of the epidermis. These infections are widespread in both temperate and tropical climates.

The name "dhobie" or "washerman's itch" has been given because the disease has been attributed to contamination of the underclothing while being washed with the garments of those who have the infection. This view probably has some foundation, but it is difficult to verify. Hebra described the condition as eczema marginatum as early as 1860 and since that time very little has been added to his clinical description. It is also known under a variety of names, some of which are the following: dermatitis bullosa plantaris, dermatitis rimosa, Hong Kong foot, eczematoid dermatitis, dermatomycosis, tinea circinata, and tinea cruris.

The organisms of tinea cruris (*Epidermophyton floccosum*) seem to be widespread if not ubiquitous. They are exceedingly common on the feet. This mycosis spreads through the inmates of schools, barracks, and gymnasiums and may be passed along by bathtubs and swimming pools. The fungi live for long periods of time in shoes, slippers, and socks, and may be acquired from towels and per-

haps by shaking hands. Some individuals seem to be more susceptible than others, and it has been suggested that differences in susceptibility may be due to different activity of the sweat glands.

The favorite site is the crotch, although the axillary region and the interdigital spaces are also frequently involved. The process starts as minute papules, but these rapidly develop and give rise to angry, red, swollen patches with sharply delimited margins. These red, festooned patches are usually limited to the perineum, scrotum, and inner surfaces of the thighs. The itching is often distressing, and many secondary infections or eczematous lesions result from the fierce scratching of the parts. If the patient goes to a cooler place the process may subside only to return when he returns to a hot, moist climate.

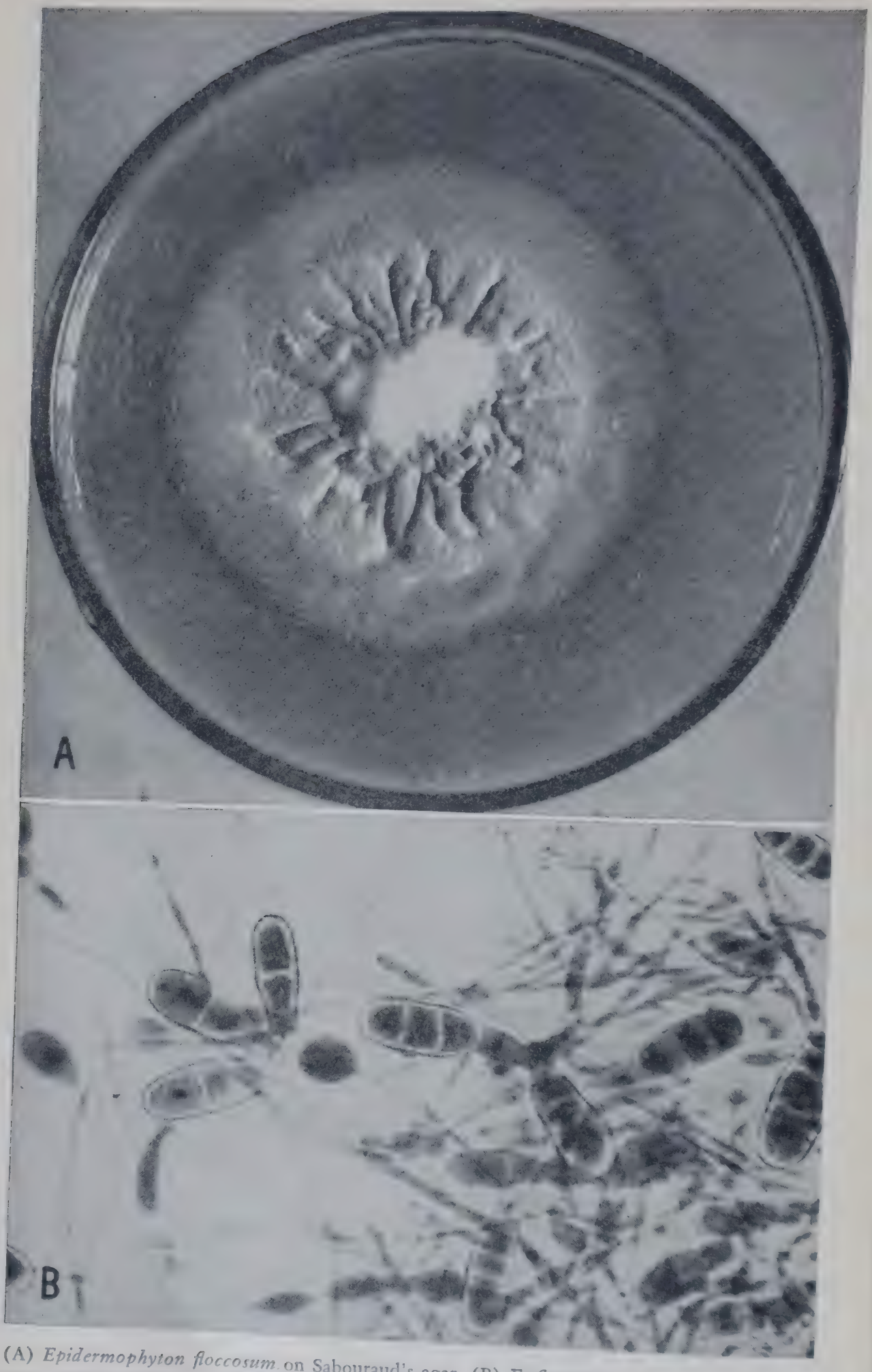
In some cases the fungus invades the regions between the toes and gives rise to a very intolerable itching and, from secondary bacterial infection, to a condition known as "mango toe." Other favorite sites for the growth of these fungi are along the outer and inner borders of the foot, in the plantar concavity opposite the instep, and in the crease between the buttocks. The lesions on the feet are well known under the designation "athlete's foot." They frequently spread to the interdigital surfaces of both hands and feet, thence spreading to the palms or soles. Eczema, pompholyx, cheiropompholyx, and a number of other names have been given to these lesions of the hand and foot.

Numerous cases of eczema marginatum and extensive lichinified eruptions of the skin have been reported in the Far East and the United States as due to the species *T. rubrum* by Lewis, Montgomery, and Hopper (1938) and by Swartz and Conant (1940).

Epidermophyton floccosum in culture develops a small, round, elevated colony, greenish-yellow in color, velvety, dry, somewhat powdery, and seldom seen over 2 cm. in diameter. Pleomorphism appears in three to four weeks, at which time the hyphae become white or gray. The culture appears like a flattened cone with a central apex, and radial folds extending to an irregular border. Microscopically *E. floccosum* shows many fuseaux, 6 to 8 $\times$ 20 to 35μ, with four or five septa. These are thick-walled and clustered.

Trichophyton interdigitale and *T. rubrum* (*T. purpureum*) are sometimes isolated from tinea cruris. *T. interdigitale* grows more rapidly than *E. floccosum*. The colony develops a central boss covered with pale buff velvet. The rest of the colony is white with no pigment on the reverse side. *T. rubrum* (*T. purpureum*) develops on the sixth to eighth day as a white, velvety colony. On the tenth to twelfth day a reddish purple color appears at the base of the colony.

The diagnosis of mycotic infections of this type should always be confirmed with the aid of the microscope. Fortunately the microscopic diagnosis is quite simple and usually successful. All that is necessary is to take a small portion of the epidermis from the periphery of the lesion or the roof of the vesicle and immerse it for a sufficient time (½ to 24 hours) in a 10 per cent solution of potassium or sodium hydroxide. This is done on a slide, the preparation being covered with a coverslip and sealed with petroleum jelly if the longer period of observation is



(A) *Epidermophyton floccosum* on Sabouraud's agar. (B) *E. floccosum:* clustered, multiseptate, clavate, macroconidia. (× 60) (Courtesy, Conant et al.: Manual of Clinical Mycology, Philadelphia, W. B. Saunders Co.)

desired. The larger the portion of epidermis taken for examination the longer the period of time required for a satisfactory clearing.

Except through secondary infection, these mycotic infections are never dangerous to life. The fungi imprisoned in the scales have been said to survive for over a year, so that a patient might be reinfected from his own slippers, shoes, etc. As the organisms multiply in the epidermis, they may live and carry over the infection to more favorable sites without ever being thrown off from the skin. Hence the advantage of using medicaments in the chronic forms of this mycosis which will keep the lesions desquamating mildly.



Tinea cruris. (From Mayer.)

Tinea Imbricata (*Malabar Itch, Tokelau*). This form of tropical ringworm is found chiefly in the islands of the South Pacific and in the Malay Archipelago. It is also found in southern China, southern India, and Ceylon. It has been reported from Colombia, Brazil, and Guatemala by Figueroa and Conant (1940). Because the disease was carried from the Tokelau Group to Samoa it is often designated as "tokelau."

Manson was the first to recognize the infection as due to a fungus which he demonstrated microscopically in the scales. He was able to transmit the disease by inoculation.

The specific cause of tinea imbricata is *Trichophyton concentricum*. It was formerly supposed that the causative fungus was *Aspergillus concentricus* but Castellani has demonstrated that fungi of this genus, when present, are merely accidental. He has isolated in pure cultures what he considers the causative fungus,



Epidermophyton floccosum, from skin scrapings. (*Left*) High power. (*Right*) Low power.







Onychomycosis, showing friable, brittle nails caused by *Epidermophyton floccosum*.

T. concentricum. Scales were treated for 10 minutes with absolute alcohol and then a single scale was placed in each of a series of tubes of maltose broth.

The fungus grows between the rete malpighii and the external epidermal layers forming a network of mycelial threads, about 3μ in width.

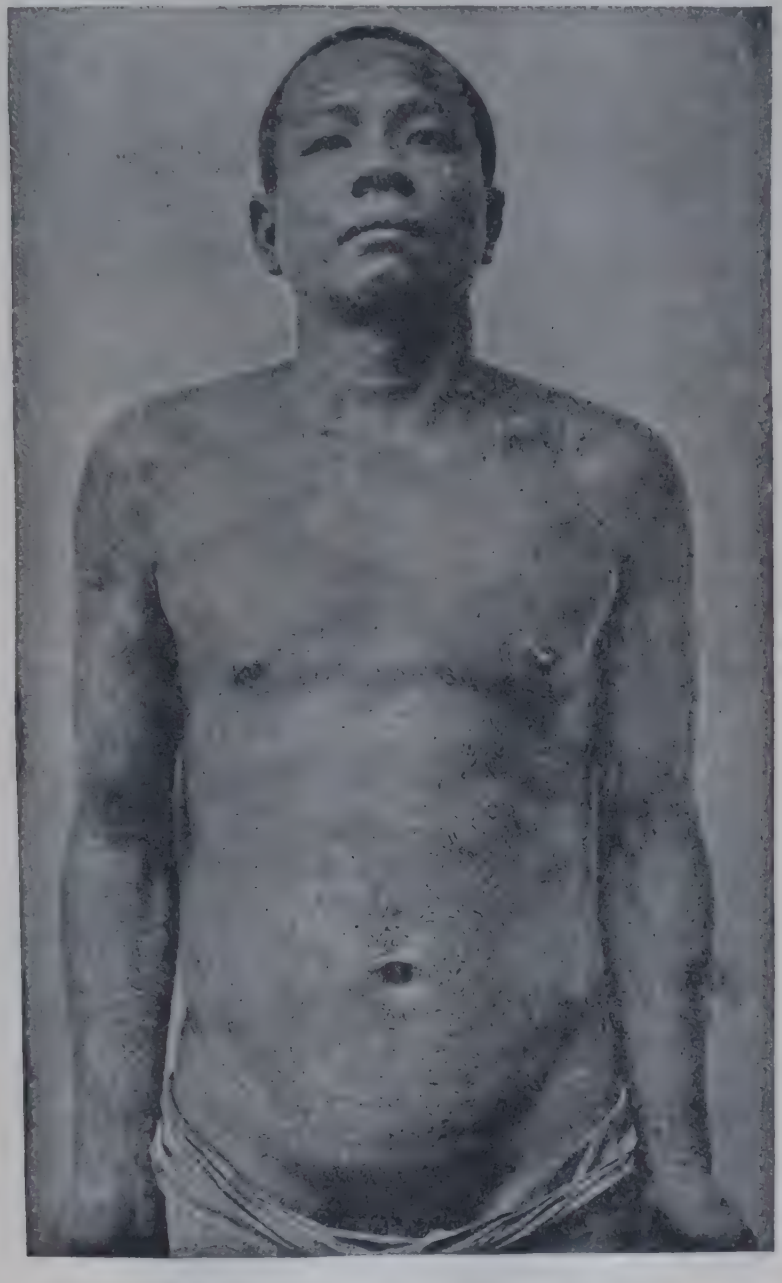
Another fungus which has been isolated from tinea imbricata scales is *T. indicum*. Inoculation of this organism in pure culture has produced the disease.

Da Fonseca has isolated, in Brazil, from a dermatosis called "chimbere" a species which he named *T. roquetti*. It is perhaps identical with *T. concentricum*.

When this disease is introduced into a country with a relative high humidity and a fairly uniform temperature, between 80° F. and 90° F., it spreads with great

rapidity. A dry climate or one showing considerable variations in temperature is not favorable for its spread.

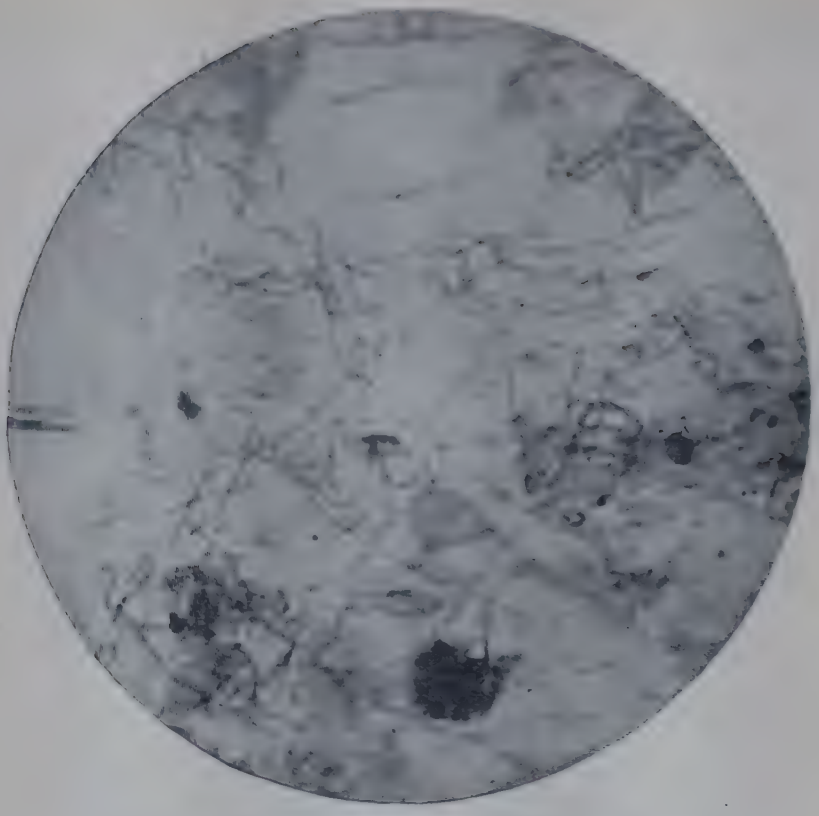
The clinical characteristic of this form of ringworm is the presence of rosette-like lesions of several concentric circles of shingle-like, papery scales which are fixed peripherally and free toward the center, thus, from its imbrications, suggesting the name given it by Manson. If one passes the finger over the affected



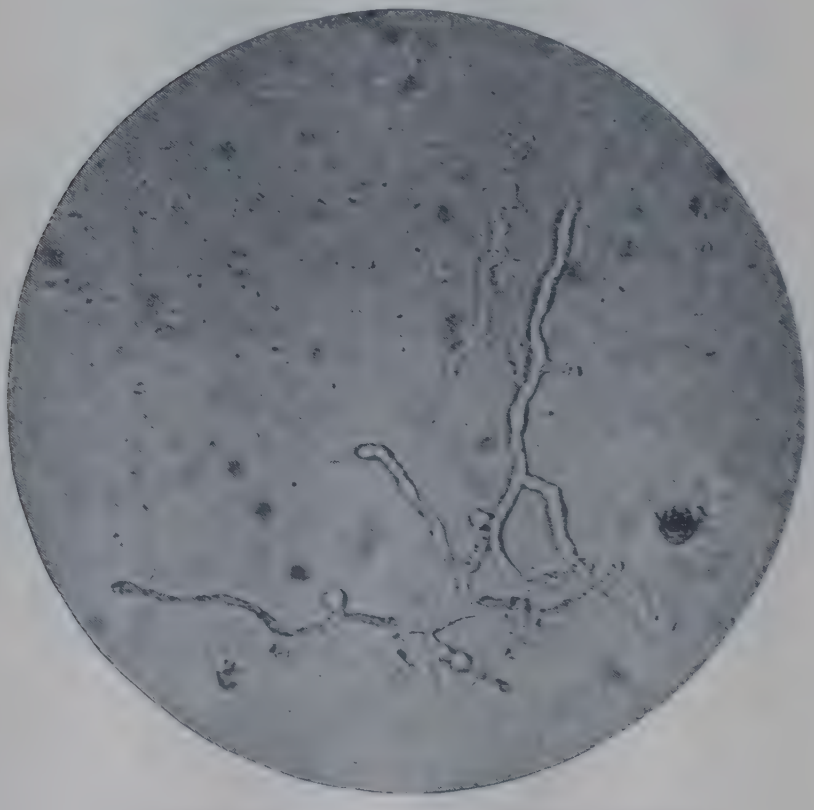
Tinea imbricata. (After Henggeler.)

surface from without inward there is no sensation of roughness but if passed from the center outward the free borders of the scales give a sensation of roughness.

When the disease is transmitted by inoculation, after about 10 days a raised, brownish spot appears at the site of inoculation. This spot increases in size until when about ¼ inch in diameter its central portion becomes detached, thus giving rise to several thin, rosette-like scales, free at the center but still attached peripherally. The fungus advances peripherally, leaving a smooth surface within.



Tinea imbricata in the skin. (Philippine case.)



Trichophyton concentricum culture. (Manila.)

A similar process again develops in the original spot, to form a circle of scales within the older and more peripheral circle. The process is repeated until several rings of scales have formed each originating from the central focus as concentric ripples on water from the fall of a pebble. The circles are from $\frac{1}{8}$ to $\frac{1}{2}$ inch apart and give a festooned appearance to the infected skin.

In infected individuals, as these circles extend peripherally they meet the peripheral rings of other circles so that various curves appear which give the general appearance of watered silk. The flaky scales are of tissue-paper thinness and are of a dirty, brownish-gray color. The general health of the patient is not affected, but the itching is very severe. There is an entire absence of inflammation about this ringworm, thus differentiating it from the more common tropical ringworms. Again the axillae and the crotch are much more rarely affected than in other ringworms, and this is also true of the face, hands, and soles of the feet. The scalp is never affected. Some claim that the fungus never invades the nails, but Manson states that this frequently occurs. The presence of the fungus in the scales treated with 10 per cent solution of potassium hydroxide differentiates the scales from those of icthyosis. Tinea intersecta is somewhat similar to tinea imbricata when first appearing, showing dark-brown patches, but it never shows the concentric rings. The ordinary ringworms present inflammatory characteristics.

Aspergilloses. Various infections of the skin (including the mycetomas) and of the ear and lungs have been reported as aspergilloses. Various species of the family *Aspergillaceae,* to which belong the very common saprophytic fungi of the genera *Aspergillus* and *Penicillium,* frequently cause contamination of bacteriologic plates and other cultures. Similarly, they may at times find a suitable medium in the skin, ears, and lungs, and many mycologists question the importance of these fungi as primary incitants of disease. The colonies of the organisms, together with those of the yeasts, *Mucors,* and spore-bearing bacteria, should be familiar to every laboratory worker.

In the family *Aspergillaceae* the genera *Aspergillus, Penicillium,* and *Scopulariopsis* will be considered. In the study of cultures and microscopic preparations of fungi it is assumed that the worker is familiar with the more or less round vesicle of *Aspergillus* which terminates the conidiophore (specialized spore-bearing stalk arising from the septate mycelium). From the vesicle, sterigmata grow which give rise to chains of conidia. The entire fruiting body, when viewed under low-power lens, gives the appearance of a round mass of black spores on a stalk. In the genus *Penicillium* the conidial chains extend from a conidiophore possessing no vesicle. The individual organism somewhat resembles the skeleton of a hand, including the carpal and metacarpal bones, as well as the phalanges (represented by the chains of spores). In *Scopulariopsis,* branching of the conidiophore is irregular. Spores, which appear in chains, are yellowish-brown and have a spiny wall.

Material for microscopic examination is abundantly at hand in the patches of molds on decaying fruits or vegetables, as well as in contaminated plates. The very common blue-green mold is *Penicillium crustaceum. Scopulariopsis brevicaule,* when grown in material containing arsenic, produces a strong odor of garlic, and is the basis of a very delicate test for arsenic (arsenic in the proportion of 1 part in 1,000,000 may be detected by this method). The very black molds will in many instances show the fruiting bodies of *Aspergillus niger. Aspergillus oryzae* saccharifies the rice starch (diastase) and, in symbiosis with a yeast, produces the

15 per cent alcoholic drink, sake, of the Orient. Other fungi of this family are of great commercial importance.

In material from sputum, pus, or skin scrapings, the morphology of the organisms does not resemble that of the fruiting bodies obtained in cultures of this material in media containing fermentable carbohydrates, hence the study of pathologic material should always include cultures.

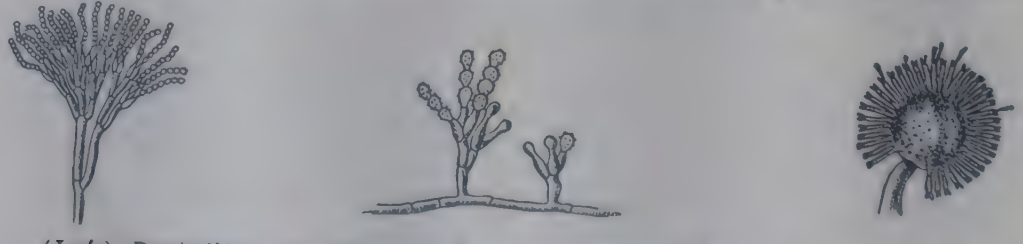
The evidence of the production of primary disease by many species of this group is not entirely convincing. *A. fumigatus,* however, can be responsible for an epizootic in pigeons and pigeon-feeders have developed aspergillosis. *A. fumigatus* has been isolated from persons having bronchopneumonia. Castellani reported species of both *Aspergillus* and *Penicillium* as a cause of bronchomycosis, but the role of *Penicillium* is doubtful. The pulmonary infection is said to resemble tuberculosis, but the patient does not seem to show an illness entirely comparable to tuberculosis. Hemorrhages are frequent, and at other times the sputum tends to be blood-streaked. Cases of aspergillosis have been reported with nasal and corneal lesions; in the latter the injury had been from scratches by plant material. *A. unguis* has been reported as the cause of mycosis of the great toe.

Mood (1938), in a study of 32 cases of otomycosis observed in South Carolina, isolated species of *Aspergillus* from 31 and a species of *Penicillium* from one. Fungi of this family were thought to be the etiologic agents of otitis externa which so severely affected many of our troops in the tropics during World War II. Studies by this author of 64 cases of otitis externa on Guadalcanal in 1943 failed to reveal fungi as the agents of this disease. In the great majority of cases *Pseudomonas aeruginosa* was considered the etiologic agent.

Scopulariopsis, which morphologically resembles *Penicillium,* has been reported from cases of onychomycosis. Markley, Philpot, and Weidman (1933) have reported the isolation of *Scopulariopsis brevicaule* from a patient with ulcerating granuloma and successfully inoculated animals with this organism. Apparently this is the first case described in the United States. *S. konigi* was reported from a gummatous lesion of the hand, and, in several instances, from trench foot.

Although tumors produced by fungi and their related organisms have been predominantly connected with species of *Actinomyces,* yet cases have been reported by Brumpt and others in which *Aspergillus* and *Penicillium* were found, and Negroni (1939) has reported the isolation of a species of *Aspergillus* in a case of mycetoma in Argentina. The evidence of the pathogenic activity of *Penicillium* is slight.

Maduromycoses (*Mycetoma, Madura Foot*). Mycetoma is the result of a fungus infection especially of the foot and more rarely of other parts of the body. It is



(*Left*) *Penicillium sp.* (*Center*) *Scopulariopsis sp.* (*Right*) *Aspergillus sp.*

characterized by swelling and hypertrophy of the tissues resulting in a deformity of the part. The infection is chronic, and recovery does not occur spontaneously.

Van Dyke Carter established the fungus nature of Madura foot, a common infection in the Madras Presidency of India. He applied the designation "mycetoma" to the disease, and Chalmers and Archibald divided mycetomas into two groups: (1) maduromycoses, characterized by granules containing large, segmented mycelium, with well-defined walls and often chlamydospores; and (2) actinomycoses, in which the granules are composed of very fine, nonsegmented filaments, with ill-defined walls and no chlamydospores. Chalmers and Archibald also recognized a paramycetoma in which the fungus did not produce granular aggregations. The maduromycoses have been separated on the basis of the color of the granules—black, white, yellow, and red. These grains may be embedded in the tissues or present in the discharge from the sinuses. Usually only one kind of fungus is found in an individual case. Brumpt, in 1906, described eight species, but in 1936 he listed 48 species as capable of producing the clinical picture of mycetoma and suggests there may be still others. The mycelium of *Madurella* is septate and branches from time to time. It is much larger than that of *Actinomyces* (usually less than 1μ in diameter whereas the hyphae of *Madurella* are always above 1μ and may reach a diameter of 8 to 10μ).

In brief, some of the organisms incriminated in the maduromycoses are: (1) black granules—*Madurella, Glenospora, Torula* (perhaps *Cryptococcus*), *Aspergillus,* and *Penicillium;* (2) white or yellowish-white granules—*Monosporium, Indiella, Sterigmatocystis, Cephalosporium,* and *Allescheria;* (3) greenish-yellow granules—*Aspergillus;* and (4) red granules—*Aspergillus* and *Rubromadurella.*

The disease was first described by Kaempfer about 200 years ago, but at the time was confused with elephantiasis. The name "Madura foot" takes its origin from the great prevalence of the infection about Madura, in the Madras Presidency of India. It is also endemic in other and widely scattered districts of India, as Delhi, Kashmir, and Rajputana. It also occurs in Ceylon and cases have been observed in Cochin China. Four cases have been reported from the Dutch East Indies by Boers, Kouwenaar, and Wolff (1938). The disease is rather widespread in Africa. Cases have been reported from Italy and Greece and from the West Indies and some of the South American countries. Sutton (1913) reported two cases from Kansas, one in a Mexican and one in a native of Texas, while Hanan and Zurett (1938) note a case from New York in a Hindu. Twining, Dixon, and Weidman (1946) report two cases (one undoubtedly contracted in the United States), one of which was caused by *Monosporium apiospermum* and the other by *Cephalosporium granulomatis.*

The disease is caused by the penetration of certain species of fungi into the tissues of the foot, as by the puncture of a thorn or through other abrasions. Rarely the hand or some other part of the body may be affected. The fungus develops in the granulomatous areas from which sinuses lead to the surface of the foot, in the discharges from which are found small granules resembling those found in the discharges from actinomycosis lesions.



Maduromycosis of foot, caused by *Monosporium sp.*

In more than 75 per cent of the cases of mycetoma the foot is the only part affected. The bony structures of the foot, as well as the muscular and areolar tissue, may undergo disintegration so that on cutting into such a foot there is nothing normal remaining—often a cheesy mass. In the early granulomatous areas there are found the typical granules surrounded by an area of mononuclear and polymorphonuclear infiltration. Giant cells are occasionally found. There is an inflammatory edema. Externally there are connective tissue cells and a fibrous wall. The blood vessels show endothelial proliferation and thrombosis. Visceral metastases do not occur.

Sporotrichosis. Sporotrichosis is a chronic infection, usually limited to the skin, subcutaneous tissues, and lymphatics, occasionally involving the muscles, bones, joints, and lungs.

This gummatous or ulcerative mycosis, which usually follows the course of the lymphatics of the forearm and arm, generally starts from a thorn prick of the hand or phalanges.

The infection is caused by *Sporotrichum schenckii* (*S. beurmanni*). Members of the genus *Sporotrichum* are widely distributed as saprophytes. The family *Sporotricheae* is characterized by branched, septate mycelium. The spores, which are generally single or in small clusters and never in chains, project from the sides of the hyphae on short sterigmata, or may be sessile. The hyphae are about 2μ in diameter. In the pus or tissues of sporotrichosis lesions one never finds mycelium, but only cigar-shaped, yeastlike bodies phagocytized in the monocytes. For diagnosis one should always culture the pus or scrapings (preferably from an



Section from a Madura foot. (After Fülleborn.)

unopened lesion). The colony first appears about the second day, and a microscopic preparation shows the narrow mycelium with spores (3 to 5μ) usually in groups at the ends of the hyphae. Optimum temperature of incubation is from 30° C. to 38° C.

The isolation of the fungus from a sporotrichosis lesion was first reported by Schenck in 1898, and later the infection was recognized as a clinical entity by Beurmann and Raymond (1903). From a standpoint of reported cases, France and



Sporotrichosis. African native.

the United States are the chief countries showing infections, but this may be due to the more frequent search for the fungus in these countries. Other cases are reported from South America and Africa.

Spontaneous infections due to *S. schenckii* have been observed in rats, dogs, and horses. The organism has been isolated from soil and plant material.

Widal and Abrami have proposed an agglutination test, and others skin tests, but the culturing technic is so simple and satisfactory that it is given preference.

Sporotrichosis when uncomplicated is rarely fatal. If untreated, however, it persists for months or years. Treatment with potassium iodide is usually effective.

Sporotricheae have been isolated from cases of tinea albigena, a skin infection of the hands and soles, later extending to the arms and legs, and quite prevalent in the East Indies. The fungus is *Aleurisma albiciscans*. A black grain mycetoma, due to *Trichosporum khartoumensis,* has been reported from the Sudan.

Chromoblastomycosis. Chromoblastomycosis is characterized by the formation of cutaneous, verrucous nodules especially on the feet and legs, although the infection occasionally occurs on the hands, neck, face, chest, ears, shoulders, and buttocks. The first case, described by Lane and Medlar (1915), was due to a fungus which was cultivated by Thaxter and named by him *Phialophora verrucosa.*

Medlar found that the cellular reaction to the fungus resembled a typical blastomycotic lesion. There was an inflammatory reaction varying from acute to chronic in type and a moderate increase in connective tissue. The process was most marked in the corium, but was also found to a slight extent intraepidermally. In the regions where the acute inflammatory reaction predominated, the exudate consisted chiefly of polymorphonuclear leukocytes and a deposit of fibrin, with an occasional eosinophil. As a general rule, in some portion of these miliary abscesses one or more microörganisms were present.

The infection usually begins on the feet or legs and is nearly always unilateral. The early lesion appears as a small papule which extends peripherally. At this stage the lesion may easily be confused with ringworm. Weeks or months later more lesions appear which are situated along the paths of the lymphatics. The infection at this time appears as warty, cauliflower-like, hard, red to grayish nodules. The papillomatous lesions may become pedunculated and ulcerate owing to secondary infection. Generally, however, the lesions are dry. The disease progresses slowly so that it may take 15 years before the entire extremity is affected. Metastases are uncommon and the infection may disappear spontaneously. Ordinarily the health of the patient is good. Diagnosis depends on laboratory examination of the tissue and the recognition and cultivation of the causative fungus.

Conant (1944) believes that the term "chromoblastomycosis" is a misnomer since it implies that the lesions have a typical color, and in addition, there are no buds or blastospores present in tissue or cultures.

Cases have been reported from Puerto Rico, the United States (notably in Texas, St. Louis, Missouri, and North Carolina), Uruguay, Argentina, Guatemala, Japan, Java, and Africa.

Carrion (1940) believes that at least three species of fungi are the etiologic

agents of this disease: *Hormodendrum pedrosoi, Hormodendrum compactum,* and *Phialophora verrucosa.* Moore and Almeida, who have studied the disease in South America and the United States, recognize as causative fungi species of *Arotheca, Phialophora, Hormodendrum,* and possibly *Trichosporum.*

Material from lesions should be examined microscopically and cultured on Sabouraud's agar at room temperature for one month before discarding. *H. compactum, H. pedrosoi,* and *P. verrucosa* appear identical in pus or scrapings. Septate, thick-walled, dark-brown bodies are present.

H. pedrosoi and *P. verrucosa* produce slow-growing, dark-brown colonies which may show considerable variation. *H. compactum* is typified by slow-growing, friable, heaped, olive-black colonies.

P. verrucosa produces flask-shaped conidiophores at the cup-shaped tips of which conidia are formed. The conidiophores may be borne laterally, terminally, or in clusters on the aerial mycelium. The presence of flask-shaped conidiophores is diagnostic.

H. pedrosoi shows much variation in conidia formation. Three types of sporulation are described. The *Hormodendrum* type is characterized by the presence of conidiophores bearing chained conidia. The conidia are olive to brown in color, single-celled, and connected to each other by thick disjuncts. The *Arotheca* type of sporulation shows conidiophores developing terminally or as single, lateral branches on aerial mycelium. The conidiophore is club-shaped from which conidia sprout on short protuberances. The *Phialophora* type is characterized by flask-shaped conidiophores.

H. compactum is differentiated from *H. pedrosoi* by the formation of terminal and lateral conidiophores from which conidia are produced in chains and are typically arranged in compact masses.

Antibiotics

Historical Note

As early as 1877 Pasteur and Joubert were aware of bacterial antagonism, having observed the phenomenon in cultures of *Bacillus anthracis* in the presence of contaminating organisms. They even suggested the therapeutic possibilities of this phenomenon.

Emmerich and Loew in 1899 used culture media containing a growth product of *Pseudomonas pyocyanea,* "pyocyanase," in the treatment of experimental anthrax. In addition to "pyocyanase," another antibiotic substance was isolated by Wrede and Strack which these investigators called "pyocyanin."

In 1913 Alsberg and Black isolated a substance which possessed antibiotic properties effective against certain Gram-negative and Gram-positive pathogens. It was given the name "penicillic acid," having been isolated from *Penicillium puberulum.*

In 1924 certain strains of *Actinomyces* were observed by Gratia and Dath to possess antibiotic properties. The substance isolated from these organisms was called "actinomycetin" and was found to be effective against Gram-positive as well as Gram-negative bacteria.

Fleming, in 1929, discovered penicillin. Strains of *Penicillium notatum,* classified by Thom, elaborated this important antibiotic. The amazing therapeutic possibilities of this substance have stimulated further intensive investigation in the field of bacterial antagonism.

Table 15

ANTIBIOTIC AGENTS OF BACTERIAL ORIGIN

(Reprinted by permission from Herrell's "Penicillin and Other Antibiotics," W. B. Saunders Co., 1945)

Antibiotic Agent	*Organism from Which Derived*	*Author Who Described Agent and Date of Report*	*Organisms Sensitive to Agent*
Pyocyanase	*Pseudomonas aeruginosa*	Emmerich and Loew, 1899	Gram-positive and Gram-negative
Pyocyanin	*Pseudomonas aeruginosa*	Wrede and Strack, 1924	Mainly Gram-positive
Tyrothricin	*Bacillus brevis*	Dubos, 1939	Gram-positive
Gramacidin	*Bacillus brevis*	Hotchkiss and Dubos, 1940	Gram-positive
Tyrocidine	*Bacillus brevis*	Hotchkiss and Dubos, 1940	Gram-positive; some Gram-negative
Bacillin	*Bacillus subtilis*	Foster and Woodruff, 1946	Gram-postive and Gram-negative

Hetherington and Raistrick in 1931 reported a species of *Penicillium, P. citrinum,* identified by Thom, as the source of another antibiotic substance, citrinum. These investigators report that the substance is primarily effective against Gram-positive bacteria.

Gliotoxin, described in 1936 by Weindling and Emerson, was next isolated from *Trichoderma lignorum,* and was found to inhibit growth of certain Gram-positive and Gram-negative organisms. In 1938 Anslow and Raistrick described fumigatin, elaborated by *Aspergillus fumigatus,* which possessed inhibitory activity against certain Gram-positive bacteria.

A substance produced by *Bacillus brevis,* gramacidin, was next reported by Dubos in 1939. Gramacidin was later called tyrothricin since it was shown that the original gramacidin was a mixture of two substances, pure gramacidin and tyrocidine. Tyrothricin is effective against Gram-positive bacteria, but practically is useful only in superficial infections, since it is highly toxic on parenteral injection.

Waksman and Woodruff in 1940 described actinomycin A and B, isolated from *Actinomyces antibioticus,* and in 1942 they reported still another antibiotic substance, streptothricin, derived from *Actinomyces lavendulae.* The growth of certain Gram-negative and Gram-positive organisms was found to be inhibited by these substances.

Claviformin, derived from *Penicillium claviforme,* was reported by Chain, Florey, and Jennings in 1942 to possess antibiotic activity against Gram-negative and Gram-positive bacteria. Claviformin was later described as "patulin."

In 1943 another substance elaborated by *Aspergillus flavus,* called "aspergillic acid," was described by White and Hill as effective against both Gram-positive and Gram-negative pathogens. In the same year flavicin, reported by Bush and Goth, and helvolic acid, described by Chain, Florey, Jennings, and Williams, both derived from *Aspergillus flavus,* were noted to possess antibacterial activity against Gram-positive organisms.

Another substance, somewhat similar to penicillin, was reported in 1943 by Philpot which he called gigantic acid since it was derived from *Aspergillus giganteus.*

Shatz, Bugie, and Waksman in 1944 described streptomycin, obtained from two strains of *Actinomyces griseus.* Streptomycin possesses little or no toxicity to laboratory animals and will be discussed in more detail.

Flavicidin, reported by McKee and Rake and Houck in 1944, was noted to be similar to penicillin in its antibiotic activity. The substance was isolated from *Aspergillus flavus.*

Undoubtedly more reports will appear in the literature describing further antibiotic

Table 16

ANTIBIOTIC AGENTS DERIVED FROM MOLDS AND FUNGI

(Reprinted by permission from Herrell's "Penicillin and Other Antibiotics," W. B. Saunders Co., 1945)

Antibiotic Agent	*Organism from Which Derived*	*Author Who Described Agent and Date of Report*	*Organisms Sensitive to Agent*
Penicillic acid	*Penicillium puberulum*	Alsberg and Black, 1913	Gram-positive and Gram-negative
Penicillin	*Penicillium notatum*	Fleming, 1929	Gram-positive and some Gram-negative
Citrinin	*Penicillium citrinum*	Hetherington, Raistrick and others, 1931	Gram-positive
Gliotoxin	*Trichoderma lignorum*	Weindling and Emerson, 1936	Gram-positive and Gram-negative
Fumigatin	*Aspergillus fumigatus*	Anslow and Raistrick, 1938	Gram-positive
Claviformin*	*Penicillium claviforme*	Chain, Florey and Jennings, 1942	Gram-positive and Gram-negative
Fumigacin†	*Aspergillus fumigatus*	Waksman, Horning and Spencer, 1942	Gram-positive
Clavacin*	*Aspergillus clavatus*	Waksman, Horning and Spencer, 1942	Gram-positive and Gram-negative
Aspergillic acid	*Aspergillus flavus*	White and Hill, 1943	Gram-positive and Gram-negative
Flavicin	*Aspergillus flavus*	Bush and Goth, 1943	Gram-positive
Helvolic acid†	*Aspergillus fumigatus*	Chain, Florey, Jennings and Williams, 1943	Gram-positive
Patulin*	*Penicillium patulum*	Raistrick and others, 1943	Gram-positive and Gram-negative
Gigantic acid	*Aspergillus giganteus*	Philpot, 1943	Gram-positive
Flavicidin	*Aspergillus flavus*	McKee, Rake, and Houck, 1944	Gram-positive

*Claviformin, clavacin, and patulin are similar if not identical.

†Fumigacin and helvolic acid are similar if not identical.

substances produced by bacteria and molds. Apparently the phenomenon of bacterial antagonism is widespread in nature, but intensive investigation will be necessary before definite therapeutic value can be ascribed to many of these antibiotic substances.

PENICILLIN

Fleming's report of the lysis of staphylococcal colonies in the vicinity of a contaminating *Penicillium* appeared in 1929. Thom classified this mold as *Penicillium notatum*. In 1941 it became apparent, as a result of investigations by the Oxford workers, headed by Florey, that the broth filtrates of this mold, containing peni-

Table 17

ANTIBIOTIC AGENTS DERIVED FROM ACTINOMYCETES

(Reprinted by permission from Herrell's "Penicillin and Other Antibiotics," W. B. Saunders Co., 1945)

Antibiotic Agent	*Organism from Which Derived*	*Author Who Described Agent and Date of Report*	*Organisms Sensitive to Agent*
Actinomycetin	*Actinomyces*	Gratia and Dath, 1924	Gram-positive and Gram-negative
Actinomycin A	*Actinomyces antibioticus*	Waksman and Woodruff, 1940	Gram-positive
Actinomycin B	*Actinomyces antibioticus*	Waksman and Woodruff, 1940	Gram-positive
Streptothricin	*Actinomyces lavendulae*	Waksman and Woodruff, 1940	Gram-negative and Gram-positive
Streptomycin	*Actinomyces griseus*	Schatz, Bugie, and Waksman, 1944	Gram-negative and Gram-positive

cillin, were of possible therapeutic value. Florey visited the United States in the summer of 1941 and with the help of Thom, principal mycologist of the U. S. Department of Agriculture at Washington, D. C., succeeded in launching a program of investigation under the auspices of the Committee on Medical Research of the Office of Scientific Research and Development. From 1941 to the present time a host of American investigators have been engaged in fundamental research which has had much to do with the successful clinical use of penicillin.

Although *P. notatum* is the common species used in the production of penicillin, according to Coghill one of the best producers of this substance is *P. chrysogenum*. There is every reason to believe that further strains of *Penicillium* will be isolated with far greater penicillin-producing potency than our known present strains.

In general, three methods have been employed for the production of penicillin—surface culture, submerged culture, and bran culture. A number of different media have been utilized, but at the present time three seem to be most effective for the production of penicillin: (1) Clutterbuck, Lovell, and Raistrick's modification of the synthetic Czapek-Dox medium, (2) amigen medium, and (3) corn steep liquor and zinc medium.

Free penicillin exists as an organic acid and reacts chemically to form various salts and esters. Two salts of penicillin most widely investigated are the sodium and calcium salts. Other salts which have been prepared include barium, potassium, magnesium, ammonium, silver, and strontium salts.

Clinically the sodium salt of penicillin has been most generally used. This salt is very hygroscopic, is easily destroyed by alteration of the pH, and is very sensitive to oxidizing agents. Heat, primary alcohols, and contact with heavy metals will alter the penicillin. Sodium penicillin, therefore, must be stored in the refrigerator at a temperature no higher than 5° C.

The calcium salt is not hygroscopic and consequently is handled with greater convenience than the sodium salt. Herrell and his associates believe the calcium salt is no more toxic than the sodium salt, and they state that in preparing oil suspensions of penicillin the calcium salt is more desirable and satisfactory than the sodium salt.

Four fractions have been found in penicillin, which have been designated as I, II, III, and K by the British, and as F, G, X, and K by the American investigators. The following nomenclature is used in the monograph on the Chemistry of Penicillin, published under the auspices of the National Academy of Sciences, based on the work of both groups: $\triangle^2$-pentenylpenicillin (I, F), benzylpenicillin (II, G), p-hydroxybenzylpenicillin (III, X), and n-heptylpenicillin (K). The antibacterial activity of these fractions, apparently, may vary. Moore (1946) points out that fraction K is not as effective against *Treponema pallidum* as fraction G. A specific lot of penicillin, therefore, with a considerable amount of fraction K and a proportionate decrease in fraction G may be considerably less potent than other lots of penicillin.

The synthesis of benzylpenicillin (although in minute yield) has been reported by de Vigneaud et al. (*Science,* 1946). These investigators report its isolation in crystalline form and positive identification with the natural product. They state, however, "Because of the obscurity of the reaction mechanism, the synthesis, at this stage of development, cannot be used as synthetic proof of structure of penicillin."

Stone and Farrell (*Science,* 1946) have reported the production of penicillin in substantial amounts in synthetic media of known composition, from which it can be isolated and purified with relative ease. The rate of growth was slower, however, and the details of the procedure required more precise adjustment than with corn steep media.

Penicillin is effective *in vitro* against the following organisms: *Diplococcus pneumoniae, Streptococcus pyogenes, Streptococcus salivarius,* micro-aerophilic streptococci, *Staphylococcus aureus, Staphylococcus albus* (some strains), *Bacillus anthracis, Bacillus subtilis, Clostridium botulinum, Clostridium tetani, Clostridium perfringens* (*C. welchii*), *Clostridium septicum, Corynebacterium diphtheriae, Streptobacillus moniliformis, Erysipelothrix rhusiopathiae, Neisseria gonorrheae, Neisseria intracellularis, Actinomyces bovis, Vibrio comma, Leptospira icterohaemorrhagiae, Treponema pallidum, Borrelia novyi,* and *Spirillum minus.*

The following organisms are apparently insusceptible *in vitro* to the action of penicillin: *Salmonella typhi, Salmonella paratyphi A, Salmonella enteritidis, Shigella dysenteriae, Proteus vulgaris, Pseudomonas aeruginosa, Pseudomonas fluorescens, Serratia marcescens, Klebsiella pneumoniae, Hemophilus influenzae, Hemophilus pertussis, Escherichia coli, Staphylococcus albus* (some strains), *Candida albicans, Candida krusei, Blastomyces, Mycobacterium tuberculosis, Brucella melitensis, Plasmodium vivax, Toxoplasma,* and *Coccidioides.*

Certain factors may influence the bactericidal activity of penicillin, not only in *in vitro* studies but also in connection with the treatment of various infections.

Variations in the sensitivity of strains of bacteria may occur. Strains which were at one time sensitive to the bactericidal activity of penicillin may, after adaptation to the substance, become "penicillin fast." The size of the inoculum and the pH of the medium may also influence the antagonistic action of penicillin. In addition, various acids, alkalies, certain alcohols, gastric acidity, heat, heavy metals, oxidation and reduction, as well as bacterial contamination may influence markedly the antibacterial activity of the drug.

Although the exact mechanism of the action of penicillin is not clear, recent investigations indicate that the substance may act as a bacteriostatic as well as a bactericidal agent. Hobby has suggested that penicillin apparently is not absorbed or destroyed in the process of bacterial inhibition. It was found that when *S. pyogenes* was inoculated in penicillin broth, the penicillin titer remained the same after inhibition had occurred. She also suggests that penicillin exerts its maximum effect only against organisms in the phase of active growth.

Unlike the sulfonamides, the action of penicillin is not inhibited in the presence of pus, products of tissue breakdown, or para-amino-benzoic acid. The presence of whole blood or serum does not have an antagonistic effect.

Herrell believes that penicillin may have the ability to block certain enzyme systems or interfere with the utilization of nutrients essential for the growth and multiplication of bacteria.

The action of penicillin *in vivo* coincides fairly well with the results obtained from *in vitro* experiments. Since it is not within the scope of this volume to include the clinical usages of penicillin, it is advisable for the reader to refer to the literature and particularly to Herrell's excellent monograph "Penicillin and Other Antibiotics."

Since no accurate chemical tests are as yet available, the methods for standardizing the potency of penicillin or for determining the concentration of penicillin in body fluids are based on tests which measure the degree of bacteriostasis by the sample. The potency of penicillin is expressed in terms of a purely arbitrary unit—the Oxford unit. It is a unit of antibacterial activity.

The test organism was a strain of *Staphylococcus aureus*. Modifications of the original Oxford or cup method of assay have appeared in the literature, some workers recommending the use of other bacterial test strains, for example, *Bacillus subtilis*. Fleming describes the following method which he calls the "hole" or "agar cup" method.

"A culture plate of agar or other suitable culture medium is planted with staphylococcus, streptococcus, or other suitable microbe. From this culture plate discs are cut out with a cork borer. The discs can readily be removed with a needle, fine scalpel, or pen-nib. The removed discs are placed in a test tube and heated in a bunsen flame to liquefy the agar. Then with a capillary pipette two drops of this melted agar are placed in each hole to seal it, and prevent any fluid which may subsequently be added from spreading beneath the culture medium. In the cup-shaped holes left in the medium are placed 2 or 3 drops of penicillin solution. This diffuses into the medium and inhibits the growth of the test organism for a distance depending on the concentration of the solution. With a reasonably potent culture of *P. notatum* the area of inhibition is about 15 mm. from the edge of the hole, or (allowing 9 mm. for the diameter of the hole) over a circle of 39 mm. in diameter."

Streptomycin*

Streptomycin is an organic base obtained by Waksman, Schatz, and associates from culture fluids of two strains of *Streptomyces griseus*. It exerts a powerful inhibitory effect on many Gram-negative as well as Gram-positive organisms, including some which are resistant to penicillin. In adequate concentration its action is bactericidal as well as bacteriostatic. It is comparatively stable, and has been obtained relatively pure as a crystalline solid. Streptomycin is hygroscopic, but if preserved dry at icebox temperatures it retains its activity for at least many months and is stable in dilute aqueous solution at room temperature for at least two weeks. Its activity toward Gram-negative bacilli is not diminished by constituents of the body fluids (diminished activity toward Gram-positive cocci has been reported) or by contaminating bacteria—no substance comparable to penicillinase has been discovered. It is partially inhibited by an acid reaction of the medium, being most active at a pH of 9.0, and by the presence of glucose and certain salts. It is inhibited completely by cysteine, but this reaction is reversible.

In general the activity of streptomycin *in vivo* parallels that *in vitro*. Pure preparations have very little toxicity, either for animals or man, but impure streptomycin may cause undesirable reactions if given intravenously. Rarely, however, untoward reactions probably attributable to the drug have been observed in man and necessitate abandonment of its use.

Marked differences in the degree of susceptibility to streptomycin are observed among different strains of the same species of organism, even those which ordinarily are most susceptible. Waksman regards susceptibility as a characteristic of a particular strain rather than of a species. A highly susceptible strain may acquire marked resistance to streptomycin with astonishing rapidity, particularly if exposed to sublethal concentrations of the drug. This tendency seems to be considerably more marked than in the case of penicillin and the sulfonamides. Such acquired resistance to streptomycin does not alter previous susceptibility to penicillin, and vice versa. The mechanism of action of the two drugs appears to be entirely different.

This acquired resistance to streptomycin (and presumably to other antibiotics also) is probably not due to a progressive alteration of the metabolic activities of the bacterial population as a whole but to a replacement of the sensitive microörganisms by an overgrowth of a few resistant individuals present before exposure to the drug. Alexander and Leidy (1947) in a study of 10 strains of type b *Hemophilus influenzae* isolated from patients before treatment found a few highly resistant organisms in all the cultures, in a proportion of from 1 : 1 billion to 1 : 14 billion. In three cases the infection became resistant to streptomycin while under treatment. Resistance was retained indefinitely when these strains were cultivated in the laboratory, and in one case a resistant strain was again recovered from the nasopharynx of the patient a year after the infection. The source of the initially resistant individuals has not been demonstrated, but they may be attributed most plausibly to a mutation.

Miller and Bohnhoff (1947) in a similar manner obtained two types of streptomycin-resistant colonies, presumably mutants, from 16 of 18 strains of meningococci which

*This section has been added by the editor, and is based largely on Waksman's publications.

were susceptible to the drug. One resistant type grew normally on the usual media, whereas the other required streptomycin for reproduction, both *in vitro* and *in vivo*.

In treatment with streptomycin, therefore, it is important practically to demonstrate the susceptibility of the strain of organism involved and to administer doses sufficiently large to insure that an effective level in the blood is promptly obtained and continuously maintained until either cure has been achieved or it is certain that the drug is ineffective. Treatment must be intensive from the start, otherwise any chance of cure by means of the drug will often be permanently lost. Susceptibility *in vitro*, however, does not insure that the drug will be effective clinically. This can be determined only by actual trial.

The procedures described for penicillin (p. 370) can readily be adapted for use with streptomycin. For assays it is customary to use as a test organism a standard strain either of *Escherichia coli* or *Bacillus megatherium*. The present unit of streptomycin is 0.001 mg. of a pure crystalline preparation and is about equal to the original unit which was the amount per 1 ml. of broth required to inhibit the growth of a standard strain of *E. coli*. It has no relation to the unit of penicillin.

Bearing in mind these limitations, the fact that susceptibility is relative, and that much of the reported work needs confirmation, streptomycin may be regarded as highly effective *in vitro* against: *Aerobacter aerogenes, Bacillus anthracis, B. subtilis,* the brucellas, *Corynebacterium diphtheriae, Salmonella typhi, Escherichia coli, E. communior, Hemophilus influenzae, H. pertussis, Klebsiella pneumoniae, Mycobacterium phlei, M. tuberculosis* var. *hominis, Pasteurella pestis, P. tularensis, Proteus vulgaris, Serratia marcescens, Salmonella paratyphi A, S. enteritidis, S. paratyphi B, Shigella dysenteriae, S. paradysenteriae, Staphylococcus albus* and *aureus* (some strains).

The following are susceptible but in lesser degree: *Diplococcus pneumoniae, Neisseria gonorrheae, N. intracellularis, Pseudomonas aeruginosa* (some strains), *Salmonella* (some strains), *Staphylococcus* (some strains), *Streptococcus pyogenes,* some strains of nonhemolyzing streptococci, *Actinomyces bovis,* and *Streptomyces* (various species); and *Borrelia novyi* and *Leptospira icterohaemorrhagiae* in experimental infections.

The following organisms (or infections) are relatively or almost completely resistant (based on clinical tests in certain instances): *Clostridium* (various species), *Mycobacterium tuberculosis* (avian type), *Malleomyces mallei, Pseudomonas aeruginosa* (some strains), *Salmonella* (some strains), *Staphylococcus aureus* (some strains), *Streptococcus faecalis* and some strains of nonhemolyzing streptococci of other types, *Vibrio comma,* various pathogenic fungi, *Treponema pallidum,* malaria, chronic ulcerative colitis, and such virus infections as have been tested.

Owing to the limited amounts of streptomycin hitherto available, its therapeutic value has not yet been precisely determined. It is highly improbable, however, that it will prove of practical value in infections with all the organisms listed above as susceptible or that it will displace penicillin in the treatment of infections which are susceptible to the latter.

CHAPTER 10

Immunity and Hypersensitiveness

Serologic Methods*

By *immunity* is meant the ability of the body to defend itself from injury by pathogenic microörganisms or by their toxic products. Immunity is, as a rule, only relative, and its effectiveness depends upon the balance between the virulence of the invading organism and the resistance of the body.

The "virulence" of bacteria, in general, depends upon their invasive power and their ability to withstand and overcome the defensive forces of the animal body, and also upon the production of toxic substances. Virulence differs greatly with different species, with different strains of the same species, and even with the same strain under different conditions of growth. Thus a highly virulent strain may lose its virulence entirely if the culture becomes dissociated. Conversely, virulence may often be restored or enhanced by successive passages through animals. Bacteria, like animals, therefore, have a protective mechanism which enables them to resist the defensive forces of the body. The nature of this mechanism is not clear, and it probably differs with different species. Bail hypothecated the development in the animal body by pathogenic bacteria of substances, "aggressins," which acted mainly by inhibiting phagocytosis. The aggressin-like action of the specific capsular substance of virulent types of pneumococci is discussed in the section on the pneumococcus. With the loss of virulence that accompanies dissociation there is also a loss of capsule formation, and the organisms become phagocytable.

Immunity. Immunity may be acquired actively as the result of an infection. In some cases, such as scarlet fever, the immunity is lifelong, whereas in others it is transient, as in pneumonia. In some diseases such as syphilis and tuberculosis immunity is usually insufficient to rid the body of infection, although it suffices as a rule to prevent superinfection (the penetration into the tissues and the growth of organisms in an animal already infected with the same species). The resistance to infection so acquired may be associated with specific antitoxic or antibacterial properties (antibodies) in the serum. As the immunity often persists long after such demonstrable activities have disappeared from the serum, it is probable that it depends primarily upon alterations in the cells—possibly the acquired capacity to produce the specific antibodies quickly and in abundance when exposure to the same infection again occurs.

Active Immunity. An active immunity may be produced artificially by the injection of sublethal doses of bacterial toxins, killed cultures, or attenuated living organisms, and even of virulent living cultures. As a rule the immunity so produced is of shorter duration than that following an attack of the disease. A high degree of immunity, however, can be secured by repeated injections, especially if

*In most instances the directions for carrying out special procedures in this section have been written or revised by Dr. Paul V. Wooley, Jr., and Dr. Sara E. Branham.

living virulent cultures are used. Hyperimmunization is brought about by the repeated injection of living virulent cultures or virus suspensions into animals which have recovered from a natural attack of the disease. The hyperimmune sera so produced possess a very high antibody content and protective power. They have been used practically in the protection and immunization of animals from such diseases as hog cholera and cattle plague.

Passive Immunity. If the serum of an immunized animal, or one which has recovered from a spontaneous infection, is injected into a normal animal, the latter may be passively protected from the infection. The degree of immunity so acquired is relatively less, and lasts only as long as the foreign serum remains in the circulation—10 days to 3 weeks.

The substances in these immune sera upon which this protective power depends are known as "antibodies." Such sera in test-tube experiments commonly show various activities toward the corresponding organism, and different antibodies have been postulated to explain each of these activities (antitoxins, agglutinins, precipitins, bacteriolysins, opsonins, etc.). It is probable, however, that these phenomena (except perhaps neutralization of toxin) are different manifestations of the activity of a single antibody. A serum which possesses the power of neutralizing a poison is commonly called an antitoxic serum, whereas one which acts more directly upon the organisms is termed an antibacterial serum.

Natural Immunity. Natural or inherent immunity may depend upon species or racial differences, or upon individual peculiarities. The inherent resistance of certain species of animals to organisms which are pathogenic for other species is usually a more complete immunity than are other types, but even this is seldom absolute. Thus the fowl, which is normally immune from tetanus, can be rendered susceptible by refrigeration, or can be killed by huge doses of the toxin. Man is naturally immune from many of the infections of animals. Natural antibodies for various bacteria occur in the serum of normal animals, usually in low concentration.

Toxins. In many cases virulence is associated with the production of poisonous products. These are of two types, exotoxins, or true toxins, and endotoxins. Exotoxins are soluble substances which are elaborated by the living organisms, and set free in the body fluids or in culture media. They are for the most part unstable substances which are destroyed by heating to 60° or 70° C., and by certain chemicals. They are highly potent poisons which act in a specific, selective manner upon special tissues. After injection into an animal a definite period must elapse before symptoms appear. The injection of the exotoxin excites the production of an antitoxin which completely annuls the action of the toxin. The ability to form toxins may be lost in dissociated cultures. They are formed most characteristically by the diphtheria bacillus, and by tetanus and some other anaerobic, spore-forming bacilli. By using improved cultural methods and virulent strains of organisms, however, the formation of potent soluble toxins has been demonstrated in a number of species whose toxic action formerly had been attributed solely to endotoxins, such as the hemolytic streptococcus, staphylococcus, and meningococcus.

The toxic action of most pathogenic organisms is due also to a substance in the

bacterial cell which is not liberated until the cell disintegrates from autolysis or from the action of bacteriolytic substances in the body. These endotoxins are less specific in their action, require no incubation period following their injection, and are relatively stable, resisting heating at 100° C. On injection into animals they stimulate the production of antibacterial sera which have little or no antitoxic neutralizing action.

Antigen. Antigen is a term applied to any substance which, when injected parenterally into a suitable animal, can stimulate the production of specific antibodies. In addition to bacteria and their toxins, practically any foreign protein, whether animal or vegetable in origin, may act as an antigen. With a few exceptions proteins from the same species of animal are not antigenic. In general only proteins possess antigenic power. Some relatively simple chemical substances, however, such as certain lipoids, carbohydrates and even drugs, which are not by themselves antigenic, may, if combined with any protein, become capable of stimulating the formation of antibodies. These react specifically with the nonprotein element of the complex, but not with the protein itself. Such partial antigens are termed "haptenes." They determine the specificity of the antigen. The polysaccharides of the pneumococcus are typical examples.

In general, antibodies are specific for the species of organism or antigen used to stimulate their formation. Frequently, however, they will also act to some extent on some biologically related species (group reactions). Thus a typhoid antiserum will often agglutinate paratyphoid bacilli to some extent, and an anti-sheep precipitating serum will also precipitate goat or ox serum. This action is due to the presence of common antigenic factors in the two species in addition to factors which are distinct and specific for each.

Occasionally an antibody will act upon some entirely unrelated species, as in the agglutination of Proteus X_{19} bacilli by the sera of patients with typhus fever. There is evidently some common antigenic factor in the *Rickettsia prowazeki* and in the Proteus X_{19}. Such antigens are spoken of as "heterophil antigens" and the corresponding antibodies as "heterophil antibodies." The Forssmann antigens constitute another widely distributed group of heterophil antigens, which contain a common lipoid haptene combined with various proteins. On injection into rabbits they stimulate the production of antibodies which cause agglutination of sheep red blood cells. The antigen has been found in the organs of various animals (horse, guinea pig, mouse, pigeon) and in various bacteria (dysentery, paratyphoid, etc.). A related antigen is present in the (unidentified) organism causing infectious mononucleosis since sera from those affected agglutinate sheep red blood cells in high dilutions.

Mechanism of Production of Antibodies. The high degree of specificity of antibodies and the capacity of the cells to produce antibody to any one of a vast number of foreign proteins which they may encounter are remarkable facts which have stimulated many hypotheses to explain antibody production. The most plausible explanation assumes that antibodies are formed from the normal globulins of the cells and that their specificity is determined by the stereoisomeric arrangement of the amino acids and polypeptid chains at the surface of the molecules. This is determined by the configuration of the antigen, the arrangement of the groups in the antibody being complementary to that in the antigen. The antigen is not used up in the process but remains in the cells, and a small amount may thus determine the production of relatively unlimited amounts of antibody.

Pauling (1940) further elaborated this theory and conceived of the superficial polypeptid chains as folded or coiled up in a pattern which is determined by the structure of the antigen and is stable for that antigen. By subjecting normal gamma globulin from ox serum for a time to a mild denaturing agent (alkali, heat) in the presence of antigen and later gradually withdrawing the denaturing agent, he and Campbell (1942) produced *in vitro* specific antibodies for methyl blue, for azo-dye protein, and for type 3 pneumococcus polysaccharide. Pauling believed that an "uncoiling" of the polypeptid chains is effected by the denaturing agent, and that these chains subsequently re-coil in a pattern which is determined by the antigen present.

It has been generally believed that the circulating antibodies are formed, in large part at least, by the macrophages of the reticuloendothelial system. Recent work (Harris et al., 1945), however, supports the view that the lymphocytes play a more direct part in their production.

Chemical Nature. The antibodies are globulins and are concentrated in the "gamma" globulin fraction of the plasma proteins as separated by the methods of Cohn and associates. Gamma globulin constitutes about 11 per cent of the normal plasma proteins and therefore contains antibodies in a concentration nearly ten times that in whole plasma. This "immune" globulin fraction of normal human plasma has been used therapeutically to abort or ameliorate certain infections, particularly measles and infectious hepatitis. Practically all sera now used for therapeutic purposes are solutions of globulin in which the antibodies have been more or less effectively concentrated and from which a large part of the inert foreign protein has been removed.

Serum Diagnosis. The diagnosis of an infection may be established by the demonstration in the patient's serum of one or more of these antibody activities against a known bacterium. Conversely, we may identify an unknown organism by observing its reaction with a known immune serum. Such sera may be obtained from convalescents, but more potent sera may be easily prepared by immunizing animals. Various other protein substances may be used as antigens, and the sera obtained are used to identify an unknown protein. Rabbits are used for the production of immune sera for diagnostic purposes. For therapeutic sera larger animals have commonly been employed, but therapeutic sera obtained from rabbits possess important advantages over those from horses and have replaced the latter for many purposes. A species of organism may possess several antigenic constituents, and, for the preparation of therapeutic sera particularly, the material inoculated must be so prepared that all the antigens are present.

Preparation of Antitoxins. These sera are used chiefly for therapy. Beginning with a sublethal dose increasing amounts of potent toxin are injected subcutaneously. In most cases it is necessary to reduce the toxicity of the preparation by the addition of antitoxin or by converting the toxin into toxoid by treating it with formalin.

Preparation of Precipitating Sera. A precipitating serum is used in medicolegal work for the identification of blood stains. A rabbit is injected intravenously with increasing amounts (2 to 10 ml.) of human serum at five-day intervals. Five injections usually suffice. A week after the last injection the titer of the serum is determined and further injections are given if necessary. The precipitate which forms is derived almost entirely from constituents of the precipitating (rabbit) serum. In the tests this is used undiluted, and titrations are carried out by adding to this increasing dilutions of the foreign serum or protein used in its production. The serum should be strong enough to give a precipitate when 0.1 ml. is added to 1 ml. of a 1 : 10,000 dilution of human serum. (See medico-

legal test for blood.) Precipitating sera may be prepared for other foreign proteins or bacteria in the same way.

Preparation of Agglutinating and Lytic Sera. Rabbits may be injected intraperitoneally or preferably intravenously. The organisms are grown on suitable solid media, and suspensions are made in salt solution. The organisms are killed by heating to 60° C. or by the addition of formalin as described under vaccines. Five injections are given at five-day intervals. The dosage to be employed varies somewhat with the toxicity of the species used. The following amounts are suitable for most bacteria, such as the typhoid-dysentery group, cholera, etc.: first dose, 1 loopful; second dose, 2 loopfuls; third dose, 4 loopfuls; fourth dose, 6 loopfuls; fifth dose, 1 agar slant. This may be followed by a similar amount of live organisms. One week after the last injection the titer of the serum is determined with blood from an ear vein, and if it is sufficiently high (1 : 1500 or over) the animal is bled from the heart with a sterile syringe and needle or other aspirating device. Twenty-five to 50 ml. of blood may be obtained in this way. The blood is allowed to clot, and the clear serum is removed. The serum is preserved by adding 0.5 per cent phenol or 50 per cent glycerin. If badly contaminated, it may be passed through a Berkefeld filter. It remains active for months if kept in the icebox. Complement disappears after a few days, and for bacteriolytic experiments it must be reactivated with fresh normal serum. In order to secure sera of maximal protective power, as with the pneumococcus, it is essential to use living cultures of high virulence for the last injections.

Antibody production varies with different rabbits, and it is desirable to immunize several at one time to obtain a potent serum. Occasionally an animal may die suddenly after an injection with symptoms resembling those of anaphylactic shock when intravenous injections are given. If intraperitoneal administration is used this rarely occurs, but the doses given should be larger and should be followed with living organisms. The method for preparing hemolytic serum is described in detail in the section on the Wassermann reaction.

Adjuvants. In the case of many antigens, the capacity to stimulate the production of antibodies and produce immunity is markedly increased if the antigen is mixed with certain adjuvant substances before subcutaneous injection. The adjuvants most frequently used are based on that described by Freund and McDermott (1942). One part of physiologic salt solution containing the antigen in solution or suspension is mixed with one part of aquaphor or falba and homogenized. To these are added two parts of paraffin oil in which there has been suspended a sufficient amount of dried tubercle bacilli which have been previously killed by heat to yield a dose of 0.2 to 0.6 mg. in 1 ml. of the mixture. This is thoroughly mixed and 1 ml. is injected subcutaneously.

Falba is a proprietary mixture (Pfaltz and Bauer, Inc., New York) described as a lanolinlike adsorption base, and is said to contain beeswax, paraffin oils, and oxycholesterin extracted from lanolin. Nonpathogenic mycobacteria may be substituted for the tubercle bacilli, but acid-fast bacteria have been found essential for the maximum effectiveness of the mixture. The mode of action of these adjuvants is not understood, but presumably a more uniform and protracted absorption of the antigen may be a factor. In animals use of these adjuvants has sometimes been followed by serious and unanticipated reactions, and their promiscuous use in man is not warranted at present.

Antitoxic Sera. Antitoxic serum combines with and neutralizes the specific toxin molecule *in vitro* and *in vivo,* so that in appropriate quantities the mixture is nontoxic. The antitoxin is produced by the body cells in response to their injury by the toxin, and is produced in such excess that free antitoxin appears in the circulation. By combining with the toxin in the circulation it prevents the toxin from further injuring the body cells. Antitoxins are also produced in response to the injections of other soluble poisons, such as snake venom and plant poisons. In

such toxin-antitoxin mixtures the toxin is not destroyed, but is rendered innocuous. For instance, if a mixture of pyocyaneus toxin and antitoxin is heated, the antitoxin is destroyed, and the mixture regains its original toxicity. (Pyocyaneus toxin differs from other toxins in withstanding a temperature of 100° C.)

Antibacterial Sera. Antibacterial sera, produced by the injection of the bacterial bodies or the products of their disintegration, may manifest the following activities: bacteriolysis (hemolysis)—lysis of the cells; agglutination—clumping of the organisms; precipitation—production of turbidity or flocculi in clear solutions; opsonic activity—alteration of the organisms so that they can be ingested by phagocytes; complement fixation; and protective power, which may depend upon these, or other properties of the serum not demonstrable at present. The extent to which these various properties may be demonstrated varies with the species of organism concerned.

Bacteriolysis. Bacteriolysis can be demonstrated by injecting cholera vibrios into the peritoneal cavity of a guinea pig together with a little cholera immune serum (Pfeiffer's phenomenon). If at intervals a drop of exudate is aspirated with a fine capillary pipet, actual disintegration of the vibrios can be observed.

The same phenomenon can be demonstrated *in vitro* if the vibrios are mixed with fresh cholera immune serum, but not if the serum is old, or if it has been inactivated by heating to 56° C. If, however, a little fresh normal serum is added (which by itself has no effect on the vibrios), the inactivated immune serum completely recovers its power of dissolving the organisms. It is said to be "reactivated." Two substances are, therefore, required for the reaction. One is a thermostable specific antibody, present only in the immune serum. The other is thermolabile, and not specific, since it is present in fresh normal as well as immune serum. Furthermore, if the cholera vibrios are removed from the immune serum by centrifugation and washed free from serum with salt solution, they will be dissolved if placed in fresh normal serum. They thus absorb from the serum the specific antibody, and become "sensitized" to the active substance in normal serum. To the nonspecific labile substance in normal serum (Bordet's "alexin") Ehrlich gave the name "complement," because it was required to complete the action of the specific antibody. To the latter, the term "sensitizing substance" was given by Bordet. Ehrlich applied the term "amboceptor" to it, because he believed that it served as a coupler to attach the complement to the cell. To the portion of the cell to which the amboceptor was supposed to become attached, Ehrlich gave the name "receptor." Although Bordet's theory corresponds more closely to modern conceptions of the nature of the process than does Ehrlich's hypothesis, the latter is useful in furnishing a simple scheme by which the details of the process can be readily visualized. "Complement" is not a simple single substance. At least five different ingredients are concerned in its activity.

If foreign red blood cells are injected into an animal, analogous antibodies, "hemolysins," are produced, and their effects are more readily studied, since the phenomenon of hemolysis is easily visible. Like the bacteriolysins, the hemolytic antibody, or amboceptor, is stable at 56° C., and may be kept for long periods.

When such a serum, inactivated by heating and suitably diluted, is added to a suspension of the homologous red blood cells, no hemolysis takes place, but the amboceptor becomes bound to the cells and sensitizes them. After the cells are washed and resuspended in isotonic salt solution hemolysis will take place on the addition of complement from some normal animal. These sensitized cells are used to determine the presence or absence of free complement in a mixture as in the Wassermann reaction and other complement-fixation tests. These reactions are quantitative, and the amount of the antigen, amboceptor, and complement must be accurately adjusted in the tests. Methods for performing these tests are given in detail in the section on the Wassermann reaction.

Agglutinins. Although Gruber and Durham first noted that cultures of the colon bacillus and the cholera vibrio would become aggregated into clumps when treated with specific serum, Widal first applied this agglutination reaction to the diagnosis of typhoid fever. In the process of agglutination *in vitro* the bacteria lose their motility (if motile) but are not killed. They may be cultivated in broth to which immune serum is added, and in this medium they develop in clumps or chains (Pfaundler's thread reaction). Agglutinins act upon dead as well as upon living bacteria.

Agglutinins, like other antibacterial antibodies, are relatively stable on standing and are not destroyed by inactivation. Complement is not necessary for the reaction. Electrolytes are necessary for agglutination; therefore the serum and bacterial suspensions must be prepared in physiological salt solution. Union of the agglutinin and the bacteria occurs without the presence of salts, but the actual aggregation involves physical changes which depend upon the action of the electrolytes.

Of great importance in the study of agglutination reactions is the recent work on variation and dissociation of bacteria. Studies have shown that many bacteria contain more than one antigenic constituent each of which may give rise to specific antibodies. In the pneumococcus, for example, there is, in addition to the protein of the bacterial body, a carbohydrate present in the capsular material, which, when combined with the bacterial protein, gives to it a type specificity. This carbohydrate alone is not antigenic, but it reacts like a haptene with the antibody produced by the combined antigen. Agglutination produced by such an antigen-antibody reaction occurs in large flakes. When a strain has become dissociated by prolonged cultivation or other means into the rough (R) colony form, the capsular material is lost, and the antigenic properties of the strain are thereby altered. Agglutinating sera prepared with these R strains are no longer type-specific, and when added to an emulsion of the organisms the resulting agglutination is of the small-flake or granular type. Such cultures also tend to agglutinate spontaneously.

In the case of a motile species like the typhoid bacillus, there are at least two antigenic substances, one contained in the flagella (the H or "flagellar antigen"), and one in the body of the bacillus (the O or "somatic antigen"). (See section on the typhoid bacillus.)

Of the other members of this group of organisms, some may possess one or another of these antigens in common. Furthermore, in some species the flagellar

(H) antigen may occur in two forms (diphasic). In one phase it is specific, whereas in the other (group) phase it is formed also in other related species. Identification then depends upon agglutination tests with sera prepared with each of these antigenic constituents.

For clinical purposes, however, it is usually possible to differentiate an organism from closely allied species by "absorption of agglutinins" from the serum. For example, a typhoid immune serum will frequently agglutinate paratyphoid as well as typhoid bacilli, but usually only in a lower dilution. An immune serum absorbed with a suspension of the homologous organism loses all its agglutinating ability for allied strains as well. If, however, it is absorbed with a heterologous strain, only the corresponding group agglutinin is removed, and the agglutinating activity for the homologous organism is but little affected. If an unknown organism, therefore, removes all agglutinins from an immune serum, we know that it is identical with the type used in its production, but if it removes only group agglutinins, leaving the specific agglutinins unaffected, it must be only a related strain. By absorbing the group agglutinins from an immune serum it can be made specific.

Diagnostic Use of Agglutination Tests. The demonstration of specific agglutinins in a patient's serum is of particular diagnostic value in cases of typhoid and salmonella infections (Widal test), brucellosis (melitensis and abortus types), tularemia, typhus fever (using Proteus X_2 and X_{19} as the antigens), Rocky Mountain spotted fever (Proteus X_{19}), and occasionally in shigella infections. Agglutinins develop in other diseases such as cholera and dysentery, but only after the acute phase of the disease is over, and other bacteriologic methods are more useful in diagnosis. The tests are performed in the following way.

Macroscopic agglutination tests. Suitable bacterial suspensions may be purchased, or they may be prepared in the laboratory. Strains of known agglutinability are grown on appropriate solid media, and emulsified in a few ml. of sterile salt solution. To this suspension is added 0.1 per cent of formalin. The tubes are then stoppered tightly and placed in the icebox for three days, after which they should be sterile. These formolized suspensions keep for months. Live suspensions may be used equally well, but are less safe to handle. In the case of the typhoid bacillus, however, the O antigen is interfered with by the formalin and it is necessary to use in addition an alcoholized suspension which contains the O antigen, and preferably also a live suspension of a strain that contains the Vi antigen. Parallel tests should also be made with the paratyphoids. When the suspensions are sterile they are diluted with salt solution to a constant density—approximately that of tube 5 of the nephelometer tubes used in preparing vaccines.

Ten small test tubes of about ⅝ inch diameter are placed in a rack. Into the first tube is pipetted 0.9 ml. of physiological salt solution, and into each of the other tubes 0.5 ml. To the first tube is added 0.1 ml. of the serum to be tested. After the contents of the tube are mixed, 0.5 ml. is transferred to the second tube, and so on through the ninth tube. Five-tenths ml. is discarded from the ninth tube. One-half ml. of the bacterial suspension is then added to each of the 10 tubes. The serum dilution in the first tube is now 1 : 20, in the second tube 1 : 40, and so on. The dilution in the ninth tube is 1 : 5120. The tenth tube is a control without serum. The tubes are shaken and placed in a water bath at 37° C. for two hours, and agglutination recorded if present. Then the tubes are placed in the icebox overnight and read the following morning. The tubes are shaken gently and the highest dilution in which definite agglutination is apparent is noted. This dilution repre-

cents the titer of the serum. In some cases, especially with the Brucella group, agglutination may be absent in the first few tubes, and appear only in the higher dilutions (*prozone-agglutinoid zones*).

In order to avoid the difficulty arising from variations in the agglutinability of different strains of typhoid bacilli, and to standardize the results of agglutination tests, Dreyer prepared "standard agglutinable suspensions" suitable for making the test, by the addition of 0.1 per cent formalin to broth cultures. These suspensions are standardized against a particular antiserum, and any increase or decrease in the agglutinability of the emulsion is expressed as an "agglutinability factor" by which a correction can be made. The end titer of a serum is converted into a "reduced titer" by dividing the dilution by this factor. Since these suspensions were first used it has been found that the use of formalin interfered with the O antigen and that only the large-flake agglutination characteristic of the H antigen occurred. If, therefore, the agglutinins in the serum of a typhoid case are mainly of the O type, as sometimes happens, this suspension may fail to detect it. For this reason an additional suspension is now made which is kept in the form of a thick emulsion in 50 per cent alcohol, diluting it to the required density with salt solution when performing the test. In using these standard agglutinable cultures for the antigen, therefore, one uses both formolized (H) antigen and alcoholized (O) antigen.

For the interpretation of the results of agglutination tests in various diseases see the sections in preceding chapters.

Tests for the identification of unknown bacteria with immune sera are made in the same way. Since the titer of these sera is higher than that usually found in a patient's serum, the dilutions may start with 1 : 50 or 1 : 100. Type determinations of pneumococci and meningococci are described in those sections.

Slide Agglutination Tests. It is often simpler to perform slide agglutination tests than the tube test. This is especially true in the grouping and typing of enteric organisms. A sheet of glass is ruled off in squares with a diamond point of ink. Loopfuls of the sera to be used are placed in each square and a small amount of the antigen is added to each. Mixing is accomplished by tilting the plate gently back and forth. Agglutination can be seen easily when the plate is held against the light. Sera for the grouping and typing of the shigella are so standardized as to be used in this technic.

Tests similar to the Widal can be performed by the slide method more rapidly than by the tube technic. The patient's serum can be diluted with a serologic pipet and special antigens ("febrile" antigens) used.

MICROSCOPIC AGGLUTINATION TESTS. These tests are less commonly used now than formerly. In this method a loopful of the bacterial suspension is mixed with a loopful of each of several serum dilutions, and hanging-drop preparations are set up. Control preparations in salt solution or in 1 : 10 normal serum are essential. These are placed in the thermostat for one hour, and examined under the high power of the microscope for clumping and loss of motility. This method is applied also to the rapid identification of colonies on culture plates.

AGGLUTINATION TEST FOR WEIL'S DISEASE. Living, four- to six-day-old cultures of *Leptospira icterohaemorrhagiae* and *Leptospira canicola* grown in Verwoort-Schüffner medium are used. Using sterile tubes, the serum of the patient is diluted with Verwoort-Schüffner buffer solution in the following manner:

Tube	1	2	3	4
Buffer	1.2 ml.	0.9 ml.	0.9 ml.	0.9 ml.
Serum*	0.3 ml.			
Dilution	1 : 5	1 : 50	1 : 500	1 : 5000

*Transfer 0.1 ml. of the serum buffer mixture from tube no. 1 to no. 2, from no. 2 to no. 3, and from no. 3 to no. 4.

Two sterile porcelain plates are used for each specimen to be examined. The tests are set up as in the following model, taking care to avoid contamination:

L. icterohaemorrhagiae			*L. canicola*		
Row 1	*Row 2*	*Row 3*	*Row 4*	*Row 5*	*Row 6*
.15 ml. 1 : 5 .15 ml. antigen (1 : 10)	.05 ml. 1 : 5. .10 ml. buffer .15 ml. antigen (1 : 30)		The same procedure as that used for *L. icterohaemorrhagiae* except for the use of an *L. canicola* antigen.		
.15 ml. 1 : 50 .15 ml. antigen (1 : 100)	.05 ml. 1 : 50 .10 ml. buffer .15 ml. antigen (1 : 300)				
.15 ml. 1 : 500 .15 ml. antigen (1 : 1000)	.05 ml. 1 : 500 .10 ml. buffer .15 ml. antigen (1 : 3000)				
.15 ml. 1 : 5000 .15 ml. antigen (1 : 10,000)	.05 ml. 1 : 5000 .10 ml. buffer .15 ml. antigen (1 : 30,000)	.15 ml. buffer .15 ml. antigen (*control*)			

The porcelain plates are covered to prevent drying and incubated for three to four hours at 32° C. or six hours at room temperature. Each dilution is then examined by darkfield for agglutination or lysis. Agglutination often occurs in the lower dilutions and lysis in the higher dilutions. Both of these reactions are specific. No reaction in the 1 : 10 dilution with positive results in higher dilutions may be observed. The material for darkfield examination is placed on a glass slide without a coverslip; the low-power objective and a compensating ocular are used.

AGGLUTININ ABSORPTION TESTS. If a sufficient quantity of bacteria is added to an agglutinating serum, the agglutinins for that particular organism are bound to the bacteria and can be removed by separating them from the serum. The exact proportion of bacteria required varies somewhat with the titer of the serum and other factors. The following method is usually adequate to effect complete absorption: A suspension of living (or killed) bacteria is centrifuged at high speed until the organisms are well packed. To 1 ml. of a 1 : 10 dilution of the serum 0.1 ml. of the packed bacteria is added. This is shaken well and incubated in a water bath at 37° C. for two or three hours. The serum is freed from bacteria by centrifugation. (The amount of salt solution introduced with the packed bacteria is too small to alter appreciably the dilution of the serum.) In making macroscopic agglutinations with this serum a control test should be set up with the species used to absorb it to make sure that absorption is complete.

Tests for isoagglutinins and isohemolysins in human blood are described in Chapter 13.

Precipitins. Precipitins can be demonstrated in the sera of animals immunized with bacteria, bacterial filtrates, or other antigenic substances, and are closely allied to agglutinins. When such sera are mixed with a clear filtrate of the homologous antigen, visible precipitates are formed. This action is due to the coating of minute particles of the antigen with the globulin with which the antibody is associated, and subsequent aggregation and flocculation of the combination. The reaction is specific for the protein used in the immunization. Group precipitins, analogous to group agglutinins, occur for closely related species of bacteria. Normal sera seem never to contain precipitins.

Clinically the precipitin test is less often used than the agglutination test. Precipitin reactions may sometimes be obtained in the spinal fluid in cases of *Hemophilus influenzae* b

meningitis when the organisms cannot be obtained in cultures. In pneumonia due to one of the fixed types the specific soluble substance (carbohydrate) may be demonstrated in the urine and in the sputum by the precipitin test with the homologous immune serum. The details of these tests are described in the sections on these bacteria.

The technic of bacterial precipitin tests is similar to that of macroscopic agglutinations, but relatively low dilutions of the immune serum must be used—undiluted, 1 : 2, 1 : 4, 1 : 8. To 1 ml. of these dilutions an equal volume of clear bacterial filtrate is added. Control tests should be made with normal serum. The test is more sensitive if the filtrate is layered on the serum.

Grouping of Streptococci (Precipitin Test). Antisera for groups A, B, and C will be sufficient for the vast majority of strains of streptococci thought to be human pathogens. The remainder of the groups can be added if necessary. Pure cultures are grown in 35-ml. amounts of infusion or extract broth until a good density is obtained, usually 24 to 48 hours. The broth is then centrifuged and the supernatant fluid discarded. To the remaining organisms 0.4 ml. of N/5 hydrochloric acid is added and check is made for acid reaction on a spot plate, using thymol blue as an indicator. If a definite acid reaction is not obtained, a drop of a normal solution of hydrochloric acid is added. The tube is placed in a water bath of boiling water for 15 minutes, shaken at the end of each five minutes, and cooled under running water at the end of the 15-minute period. Using phenol red in a spot plate, enough N/5 sodium hydroxide is added to bring the reaction to 7.6. The tube is then centrifuged; the resulting supernatant fluid is to be used for testing.

A small conical tube is prepared by slightly drawing 7-mm. glass tubing in a flame. The drawn tubing is cut in the center to make two tubes. The narrow end of each tube is sealed into a knob to prevent the tube from slipping through the rack. The 7-mm. diameter is retained at the open end to facilitate pipetting, and the lower end has a bore of about 3 mm., holding 0.1 ml. in a column 8 to 10 mm. high. Good results have been obtained by putting 0.05 ml. of the extract into the tube first, and then 0.05 ml. of serum. Since the serum is heavier, it sinks below the saline extract and forms a layer with about the right amount of mixing. With the usual technic employed in ring tests of placing the serum in the tube first, too narrow a ring is formed due to insufficient mixing in a tube of such small caliber. If, owing to an air bubble, the fluid fails to lodge at the bottom of the tube, no attempt is made to shake it down. The result can easily be read at any level, and shaking is apt to interfere with the formation of a good plane of junction. Positive reactions are usually obvious at once, and can be safely recorded after one-half hour at room temperature. In order to save serum exceptionally potent antisera may be diluted, but not beyond the point where an immediate heavy ring-reaction is obtained with extracts of the homologous group.

Medicolegal Test for Blood (Uhlenhuth). The anti-human serum is titrated in the following way. In each of four small test tubes place 0.1 ml. of concentrated serum. Over this, layer 0.9 ml. of the serum to be tested for, in dilutions of 1 : 100, 1 : 1000, 1 : 10,000, and 1 : 20,000. If the serum is adequate in strength, a definite white ring will appear at once in tube 1, after one minute in tube 2, after four minutes in tube 3, after about six minutes in tube 4. The serum must give negative reactions (throughout a period of observation of 20 minutes) with salt solution, and with 1 : 1000 dilutions of serum from other unrelated animals.

The dried blood to be examined is scraped off, or if on a fabric the spot is cut up, and the material extracted in salt solution for several hours. A portion of the unstained fabric is treated similarly as a control. The extracts are filtered until clear, and a portion is diluted until it corresponds in protein content roughly to a 1 : 1000 dilution of blood (about 0.01 per cent protein). This can be estimated by shaking the tube vigorously and noting the amount of froth. A 1 : 1000 dilution shows a moderate amount of froth which persists from three to five minutes. A 1 : 10,000 dilution does not froth. If sufficient

material is available, the tests for estimating the quantity of albumin in the urine may be employed to check the dilution.

Over 0.1 ml. of the appropriate immune serum layer 0.9 ml. of the suspected extract properly diluted. If positive a ring will appear in one to two minutes. As controls the unstained fabric extract is tested similarly with the same immune serum, and the extract of the unknown blood with normal rabbit serum, and preferably with two or three antisera prepared with blood of other unrelated species of animals. If these controls show no ring after 20 minutes a positive reaction is dependable. An anti-human serum will also react with the blood of most monkeys, but not of any other animals.

The test is for protein and not for blood as such. (See p. 812 for tests for hemoglobin and its derivatives.)

Opsonins. Phagocytosis is one of the most important defensive mechanisms of the body against the invasion of bacteria. There are two kinds of phagocytes in the body—the microphages or polymorphonuclear leukocytes; and the macrophages which include the monocytes of the blood and the fixed tissue (reticulo-endothelial) cells. In ordinary bacterial infections the former play the predominant role. The latter are chiefly concerned in the phagocytosis of animal cells and protozoa. Although other observers had previously noted the presence of substances in immune sera which stimulated phagocytosis, Wright and Douglas in 1903 first demonstrated that these substances, which they designated opsonins, acted directly upon the homologous bacteria, rendering them susceptible to phagocytosis. These opsonins unite with the bacteria just as do other antibodies, and are not removed by washing the bacteria. The opsonins in immune sera (bacteriotropins of Neufeld) are specific for the species used in immunization, and may be absorbed from the serum. Opsonins, active on some of the less virulent types of bacteria, occur to some extent in normal serum, but their activity is slight compared with that of the immune opsonins. They may be increased in the serum of convalescent patients. The presence of complement is apparently not essential for phagocytosis, but it accelerates the reaction greatly. Dissociated forms are more easily phagocyted than are smooth types of the same organism.

Many attempts have been made to estimate quantitatively the opsonic activity of human serum, both as a means of diagnosis and as an aid in controlling treatment, but no method has proved to be of practical value clinically. The procedure has been revived recently, however, for the diagnosis of brucellosis.

Opsonocytophagic Index. This test at the present time is limited in scope to the study of brucellosis.

To a vial containing exactly 0.2 ml. of 20 per cent sodium citrate 5 ml. blood are added. This blood must be used within six hours following collection and should be kept cool during this period. A dense suspension of a 24-hour growth of smooth brucella culture is then made in saline. It does not matter which of the brucella types is used as long as the strain is smooth. It is important to employ a dense suspension, comparable to what would be the sixteenth tube in the McFarland scale.

In a small serologic tube, place 0.1 ml. of the blood, which should be thoroughly agitated before the sample is withdrawn, and 0.1 ml. of the bacterial suspension. Mix thoroughly and place in a 37° C. water bath for 30 minutes. The contents of the tube are not agitated during incubation but at the end of this period they are mixed thoroughly and drops are withdrawn to clean slides. Films are prepared on the slides by dragging the blood along on the edge of another slide, exactly as is done in making blood smears. The films are dried rapidly and stained for two minutes in toluidine blue, made by dissolving 0.5 Gm. of the stain in 10 ml. ethyl alcohol and adding 3 ml. phenol and enough water to make 100 ml. The slides are rinsed in water and examined with the oil-immersion lens.

Twenty-five consecutive polymorphonuclear cells are examined for the degree of phagocytosis and a record is made as follows:

No phagocytized organisms	Negative
1 to 20 bacteria in cell	Slight
21 to 40 bacteria in cell	Moderate
Over 40 bacteria in cell	Marked

In rendering a report, the 25 cells are listed only according to the amount of phagocytosis, such as: 10 cells slight, 10 cells moderate, 5 cells marked.

Bacterial Complement Fixation. Immune sera contain antibodies which, in the presence of the corresponding antigen, combine with complement and, in appropriate quantities, remove it from the mixture. The presence or absence of complement in the mixture is then determined by the addition of a hemolytic system (red blood cell suspension, sensitized with its specific antiserum, which has been inactivated to remove complement). Such a test is performed exactly as is the Wassermann test, with bacterial preparations as the antigen.

This test has been used chiefly in the diagnosis of gonorrhea, glanders, and brucellosis and is valuable in rickettsial infections. It is of some value in tuberculosis, but the reaction is usually negative in early cases. A positive reaction may be obtained in a number of other infectious diseases including those due to filtrable viruses, but for practical purposes cultures or other immunity reactions are usually less complicated and time-consuming. The test is also useful in the diagnosis of some of the animal parasitic diseases, especially echinococcus infection, and infections by various trematodes and trypanosomes.

In performing the test, titrations of the antigen must be carried out as in the Wassermann reaction to determine: (1) hemolytic activity (if it is hemolytic in a dilution of more than 1 : 5, discard); (2) binding power, or ability to fix complement in the presence of its antiserum; (3) anticomplementary action, or ability to inhibit the action of complement in the absence of serum. For the actual test one should use not more than one-half of the maximum quantity which just fails to be anticomplementary.

Various methods for preparing the antigen have been advocated.

1. The growth on agar, or special media, is emulsified in salt solution, as in the preparation of vaccines. The suspension is heated at 60° C. for an hour, and then standardized by counting or by the use of the nephelometer tubes. For the gonococcus test 4 billion per ml. makes a satisfactory antigen. This may be preserved by the addition of 0.25 per cent trikresol or 0.5 per cent phenol. It is important to prepare the gonococcus antigen from a mixture of a number of strains. For glanders one may use a 72-hour culture in glycerin broth, sterilized at 60° C. for two hours.

2. Besredka and Gay prepared their antigen by precipitating the saline bacterial suspension with an equal amount of absolute alcohol. After centrifugation, the supernatant fluid is pipetted off, and the sediment dried *in vacuo* over sulfuric acid. The dried sediment is made into a 2 per cent suspension with isotonic salt solution. For use this stock solution is diluted. There are also methods in which the bacterial sediment is frozen with carbon dioxide snow, and then triturated with crystals of sodium chloride so as to make an isotonic saline emulsion.

3. An agar growth is washed off with 50 per cent alcohol and the suspension centrifuged. The supernatant alcohol is pipetted off. Fifty per cent alcohol is added; the mixture is allowed to stand for 30 minutes at 37° C. and then centrifuged. The supernatant alcohol is removed. Ether is added and, after shaking, the suspension is allowed to stand one hour at room temperature. If the ether is yellow, fresh ether is again used for washing. Then the suspension is allowed to stand until the sediment is perfectly dry. For use, 1 Gm. of the powder is suspended in 200 ml. of physiological salt solution.

Protection Tests. The protective power of a serum depends upon those properties which can be demonstrated *in vitro,* and probably also upon other factors which cannot be determined at present. For this reason the most accurate means of ascertaining the therapeutic value of an immune serum is by testing its ability to protect animals from infection. Meningococcus, pneumococcus, and dysentery sera are standardized by protection tests.

Hypersensitiveness

Hypersensitiveness is defined by Zinsser as "an increased specific reaction capacity in an individual, man or animal, to a substance which, in a normal individual of the same species, produces little or no reaction."

The mechanism of the various manifestations of hypersensitiveness is not thoroughly understood, and in consequence the terminology applied to the different phenomena has become confused. Coca and others make a sharp distinction between, on the one hand, "anaphylaxis," in which an antigen-antibody mechanism is demonstrable, and on the other hand, the group of reactions commonly termed "allergies" or "idiosyncrasies," in which this mechanism is not so clearly apparent. In this latter group are included the manifestations of hypersensitiveness to pollens, certain drugs, bacteria, foods, etc. Wells, on the contrary, believes that sufficient relationship exists between these phenomena to permit grouping all manifestations of altered reactivity together under the term "allergy." Zinsser likewise believes that the distinction between anaphylactic phenomena and allergies is artificial, and is convinced that all forms of hypersensitiveness are fundamentally identical.

For convenience in discussion, however, these manifestations may be grouped into (1) anaphylaxis in animals and in man; and (2) hypersensitiveness (allergy, atopy) to pollen and other inhaled proteins, foods, drugs, and bacteria.

Theobald Smith, studying the effects of diphtheria antitoxin in guinea pigs, and Arthus by injecting rabbits with horse serum, observed that the animals were rendered susceptible to a second injection of small amounts of the serum after an incubation period of a week or more. Various authors, notably Rosenau and Anderson, extended these observations and found that the reaction could be produced by the reinjection of a great variety of proteins, and that it is specific for the particular protein used.

Anaphylactic Shock. If a guinea pig is injected parenterally with even very minute amounts of horse serum (0.0001 ml. or even less), after an interval of 10 days a condition of anaphylactic shock may be brought about by the injection of somewhat larger amounts (0.01 to 0.3 ml. intravenously or intracerebrally, or 1 to 5 ml. subcutaneously). It is assumed that the primary injection, at the end of the requisite interval, has sensitized the body cells to the particular alien protein introduced, since the administration of large amounts of serum does not affect a guinea pig on primary injection. In guinea pigs symptoms are produced within a few minutes of the second injection. Restlessness, scratching of the nose, and sometimes sneezing, appear first. Soon signs of great respiratory embarrassment occur, which are due to spasm of the bronchial musculature, and at autopsy there is a characteristic inflation of the lungs. Convulsions may occur and the animal may die within a few minutes. If the shock is not immediately fatal, the body temperature may drop abruptly 5° or even 10° F. It has been shown that during

the shock the complement in the blood is diminished, and it is believed that this plays a part in the reaction. The coagulability of the blood is diminished, probably by liberation of heparin. It is believed that the symptoms are due to liberation of histamin or a similar (H) substance from the tissue cells.

The phenomena differ in different species of animals, but they are always the same in any one species regardless of the nature of the antigen. In rabbits there are circulatory disturbances with a fall in blood pressure, spasm of the arterioles in the lungs, and marked distention of the right side of the heart. In the dog there is engorgement of the hepatic and splanchnic circulation with an extreme drop in blood pressure.

Desensitization. If an animal is reinjected during the incubation period, before sensitiveness has developed, no reaction will take place, and the animal is desensitized temporarily to the subsequent injection. Similarly, if an animal recovers from an anaphylactic shock it is for a time desensitized. By repeated injection of the antigen in increasing amounts, beginning with a dose too small to induce symptoms, an animal may be desensitized also, and rendered insusceptible to amounts which would otherwise produce a fatal anaphylactic shock.

Passive Sensitization. When serum from a sensitized animal is injected into a normal animal, even in quantities as small as 0.5 ml., the latter is also rendered sensitive, but only after the lapse of a certain period of time, usually four hours. It is assumed that this period is necessary for fixation of the antibody to the cells. This passive sensitization lasts only about two weeks. The offspring of an animal sensitized before or during pregnancy is passively sensitized through the placental circulation.

In this type of passively induced hypersensitiveness to foreign serum there is obviously some substance present in the serum which is capable of altering the cells of a normal animal so that they become sensitive. In other types of hypersensitiveness in human beings, such as hay fever or food allergy, it is not generally possible to demonstrate by transfer to animals a sensitizing substance in the serum. It is, however, possible to produce a passive local hypersensitiveness in other human beings by intradermal injection, and it has been suggested that the inability to sensitize animals passively with human serum is due to species differences in the tissue proteins.

The substances present in the serum of sensitized animals which bring about passive sensitization are often termed "anaphylactic antibodies." The nature of these antibodies is not entirely clear, but there is reason to believe that they may be identical with ordinary antibodies. Precipitins can be demonstrated in the blood of animals sensitized with horse serum, and also in the blood of human beings suffering from serum sickness after a therapeutic injection of horse serum.

Hypersensitiveness to Homologous Proteins. Although as a rule animals cannot be sensitized to proteins derived from the same species, in certain special instances this is possible. In most cases the tissues with which this has been accomplished are derived from the ectoderm. Thus a guinea pig can be successively sensitized and later fatally intoxicated with extracts of the lens from its own eyes.

Hypersensitiveness can also be produced to tissues of the central nervous system. Rivers and Schwentker (1935) produced extensive disseminated demyelinating lesions in the brains of monkeys by a protracted series (46 to 85) of injections of normal rabbit brain. Subsequent investigations showed that such procedures stimulated the production of antibodies and the development of hypersensitiveness which were specific for the tissue

but not for the species of animal from which the tissue was obtained. More recently Morgan and independently Kabat et al. (1946, 1947), by utilizing an adjuvant of the Freund type, readily produced an extensive disseminated encephalomyelitis in monkeys by a few injections of homologous brain tissue. These lesions closely resembled in character and distribution those found in multiple sclerosis and acute disseminated encephalomyelitis in man. It has been suggested that the lesions in these diseases may be due to a local allergic reaction following autosensitization, an "allergic encephalitis."

It has also been suggested that the encephalitis that occasionally follows the inoculation of rabies vaccine may be due to sensitization to brain tissue present in the vaccine.

Tissue Hypersensitiveness. Because a period of incubation is required to bring about passive as well as active sensitization and because animals remain actively sensitive after their serum has lost the power to convey sensitiveness passively to other animals, it is generally accepted that the phenomena causing the anaphylactic reaction occur within or upon the tissue cells and not primarily in the blood stream. The symptoms of shock are believed to result from the interaction within the cells of the foreign protein and the specific antibodies which are, in part at least, precipitins. The phenomena of hypersensitiveness and anaphylactic shock are, therefore, primarily cellular rather than humoral.

Relation of Hypersensitiveness to Immunity. It is customary to include under "immunity" those forms of altered reactivity of the tissues which are obviously beneficial to the animal, and to class under "hypersensitiveness" or "anaphylaxis" those which are immediately harmful. The fundamental processes which give rise to these reactions, however, are similar in many ways. Both depend upon the increased capacity of the body cells to fix and disintegrate foreign protein which has gained access to the tissues. Many investigators have believed that whether this is beneficial or harmful depends in large part upon the nature and quantity of the foreign protein present. If the latter be in the form of a pathogenic microörganism—relatively minute in quantity but capable of indefinite multiplication—its immediate destruction and disintegration is life-saving, and the animal is "immune." If it be a relatively large quantity of foreign serum, e.g., which cannot increase in quantity in the body, its accelerated disintegration affords no distinct advantage to the animal, but this may abruptly liberate such a large quantity of toxic disintegration products that the animal is acutely poisoned; it is "hypersensitive" or "anaphylactic." The latter occurs practically only under artificial or experimental conditions, but the penetration of microörganisms into the tissues is a very frequent occurrence. Under natural conditions, therefore, this increased reactivity of the tissues might be expected usually to be beneficial to the animal.

The view that hypersensitiveness is a necessary or useful manifestation of the immunity reaction, however, has been questioned seriously, particularly by Rich. He has pointed out that although as a rule an animal which is immune is also hypersensitive to the organism in question, this is not necessarily the case. Immunity without hypersensitivity may be obtained in some instances by administering the injections intravenously or by carrying out desensitization procedures during or after completion of the immunization. Animals may gradually lose their hypersensitivity but retain their immunity. Furthermore animals may be effectively immunized passively without becoming hypersensitive. Rich found no parallelism between the degree of hypersensitiveness and the titer of protective antibodies, and has advanced evidence to show that the hypersensitive reaction accelerates rather than restricts dissemination of bacteria in the tissues. He concludes that in many cases the severe local injury to the tissues and violent systemic reactions which accompany hypersensitiveness are undesirable and harmful, and that "up to the present, hypersensitive inflammation has not been shown to be necessary for the successful operation of acquired immunity at any stage of any infection under any circumstances whatsoever."

Hypersensitiveness in Man. Human beings are much less susceptible to acute anaphylactic shock than are guinea pigs and most of the lower animals, and, there-

fore, there is relatively little danger in the administration of a second injection of therapeutic serum even after the period of anaphylactic incubation. Most serious reactions have occurred following the primary injection, particularly in asthmatics and in those who are sensitive to horse emanations. In these individuals deaths have occurred within a few minutes after a single injection of serum with symptoms of respiratory embarrassment and cyanosis. Boughton has reported the death of a man who was given 1 minim of horse serum intravenously to desensitize him for "horse asthma." However, Park has estimated that alarming symptoms occur in only 1 in 20,000 individuals and fatal reactions only in 1 in 50,000.

Before any therapeutic serum is administered, therefore, it is important to determine whether or not an individual is sensitive to horse serum, both when there is a history of previous serum administration and particularly if the person has had asthma or any form of protein hypersensitiveness. The existence of hypersensitiveness may be detected by means of the intradermal or ophthalmic test.

Ophthalmic Test. A drop of horse serum diluted 1 : 10 is instilled into the conjunctival sac. If the individual is sensitive, an inflammatory reaction occurs within 15 to 30 minutes. If severe it may be controlled by the instillation of 1 : 1000 adrenalin solution. This test is less sensitive than the cutaneous tests, and if positive great care must be used in administering serum.

Intracutaneous Test. The serum is diluted 1 : 10 with salt solution, and 0.1 ml. is injected intradermally. If sensitiveness exists an urticarial wheal will develop within 10 or 15 minutes, the extent of which gives a rough indication of the degree of sensitiveness. This is a sensitive test, and many individuals give a slightly positive reaction who do not develop any systemic reaction following the serum administration. If the reaction is negative, it is generally safe to proceed with the treatment.

Desensitization. Even in persons giving no reaction to the intracutaneous test it is desirable to give a preliminary subcutaneous injection of about 1 ml. one-half hour before starting intravenous administration of serum. In individuals giving a marked reaction to the intracutaneous test or any reaction to the ophthalmic test, preliminary desensitization should be carried out before giving the major part of the dose. This is done by beginning with a small subcutaneous dose of 0.1 ml. diluted with salt solution, and doubling the amount every half hour until 1.5 ml. have been given. If no reactions have occurred it is usually safe to give the balance of the dose intramuscularly. If intravenous therapy is to be used, one may then give 0.1 ml. diluted with 2 ml. of salt solution slowly into a vein, repeating with doubled doses every 20 minutes until the full amount is given. The rate of increase in the doses must depend upon the individual case.

It is essential to have available a 1 : 1000 *solution of adrenalin* to control any untoward reactions such as itching, tingling, urticaria, difficulty in breathing, cyanosis, circulatory symptoms, or lumbar pain. If such occur, 1 ml. should be given immediately and repeated as often as necessary. It should be remembered that its action is transitory, and anaphylactic symptoms may recur after the immediate effects have worn off. In severe reactions it may be necessary to repeat the dose every 10 to 20 minutes for several hours. Hurst (1934) injects 3 minims at the start and 1 minim every one-half to one minute until the reaction is over (the "continuous method"). In one of his cases this was kept up for one and one-half hours, and he believes that by this method death could be prevented in all cases of anaphylactic shock. If the serum has been injected into the tissues of the arm or leg, a tourniquet should be applied proximal to the site of injection; and after the acute symptoms have subsided it should be loosened cautiously, for brief intervals only, until the reaction is over.

In an anaphylactic reaction the manifestations may be generalized, or the symptoms and

pathologic lesions may be limited largely to one tissue or organ which has been termed the *shock organ*. This may be, for example, the respiratory mucous membrane and bronchial musculature (hay fever, asthma), the skin (urticaria), the gastrointestinal tract, or the walls of the arterioles (rheumatic fever, periarteritis nodosa, if the allergic nature of these lesions is conceded).

Serum Sickness. In some individuals a therapeutic injection of foreign serum is followed after from 8 to 12 days (delayed reaction) by a characteristic group of symptoms. There is usually an erythematous rash or urticaria starting first at the site of inoculation, and later becoming generalized. Fever, joint pains, and generalized glandular enlargement are common. These symptoms occur in about 10 per cent of the cases when less than 10 ml. are given, and in about 90 per cent when more than 50 ml. are used. They are less frequent when concentrated sera are injected. A subsequent injection of the same protein if given within a few weeks causes the same symptoms, usually within 24 hours (immediate reaction). If several years have elapsed between injections, symptoms appear after three to seven days (accelerated reaction).

Von Pirquet and Schick have ascribed these symptoms to a reaction between the horse serum which is still present in the circulation of the patient and the homologous antibodies which are being actively formed in the tissues. Precipitins for horse serum have been demonstrated in the blood of patients with serum sickness, and conditions are analogous to introducing new serum into a sensitized individual. Both antigen and antibody are present in the blood at the same time. Zinsser regards serum sickness as "the characteristic manifestation of true protein anaphylaxis in man."

Hypersensitiveness to Substances Other than Serum. Hypersensitiveness occurs to other substances such as pollen, animal emanations, and other inhaled protein material, foods, drugs, and bacteria.

Coca and others believe that this form of hypersensitiveness is inherited and exists naturally, in human beings only, and not as the result of artificial sensitization. Zinsser and others, on the other hand, believe that the hypersensitiveness results from contact with the antigen through the respiratory and alimentary tracts and perhaps through the skin. They point out that although hypersensitiveness tends to occur in certain families, it is not to the same protein as a rule, and conclude that it is the capacity for becoming sensitized that is inherited.

Passive Transfer. This type of sensitiveness cannot be transferred passively to animals except in isolated instances. In 1921, however, Prausnitz and Küstner found that by injecting the serum of a sensitive individual intradermally into a normal person a "local hypersensitiveness" could be produced. If, after from 4 to 12 hours, the homologous antigen was injected into the same area, a definite local reaction developed. Injections into other areas produced no reaction—only the cells in the neighborhood of the primary injection were sensitized. This local hypersensitiveness lasts several weeks. Walzer has demonstrated that when the serum of certain food idiosyncratics was injected intradermally into normal individuals and these subjects were fed the food to which the idiosyncratics were sensitive, reactions appeared in these injected areas. In the serum of the sensitive individuals,

therefore, are substances analogous to, if not identical with, antibodies to which Coca has given the name "reagins."

HAY FEVER. Hay fever is due to an allergic sensitiveness to pollens of certain plants. The flowering period of these plants determines the seasonal prevalence of the disease. Only those plants whose pollen is air-borne are of clinical importance.

Most of the cases occurring in the late spring and early summer, both in this country and abroad, are caused by pollens from the different grasses. Plantain is also frequently concerned at this season. Cases occurring in the early spring are due to the pollens of various trees. In this country the type occurring in the late summer and early fall is due most commonly to the ragweeds. In the western states sage brush sensitiveness is common at this season. Other pollens may be responsible for individual cases, and one patient may be susceptible to several different pollens. Hypersensitiveness may be detected by means of skin tests with extracts made from different pollens, and desensitization by the injection of increasing doses of the reacting extract is often successful in relieving symptoms. Sensitiveness usually returns, however, after the treatment is stopped.

Extracts of the various pollens for testing and for treatment may be purchased, or they may be prepared by extracting the pollens in many different ways. A solution containing two parts of glycerin and one part of saturated salt solution, or Coca's solution (NaCl 5.0 Gm., $NaHCO_3$ 2.8 Gm., phenol 4.0 Gm., water 1000 ml.), may be used. Five Gm. of the pollen are washed with ether and extracted with 100 ml. of this solution, and allowed to stand four days, shaking frequently. The solution is then passed through a Berkefeld filter. The extract is standardized by determining its nitrogen content. The Folin-Wu method for blood nitrogen determination may be used, taking a quantity of extract containing about 0.15 mg. of nitrogen. This solution should contain about 0.5 mg. of nitrogen per ml., or 50,000 of Noon's units. (One unit is the amount of extract derived from one millionth of a gram of pollen, and it contains in the case of timothy pollen 0.00001 mg. of nitrogen.) Extracts of other species of pollens may contain different amounts of nitrogen, but as their allergenic activity varies directly with their nitrogen content it is customary to standardize them on this basis.

Dilutions of the stock solution from 1 : 10 to 1 : 10,000 are used for testing and for treatment. To test, an intracutaneous injection of 0.025 ml. is given, usually of the 1 : 5000 dilution first, and if reaction is negative, with 1 : 1000. In some cases 1 : 100 may be necessary to bring out the reaction. A positive reaction is indicated by the appearance, in from 5 to 30 minutes, of an urticarial wheal surrounded by an area of erythema, and in the more marked reactions by "pseudopods." Tests may also be made by applying stronger solutions to a scratch. In a few individuals who are undoubtedly hypersensitive, negative skin reactions are obtained. In such cases the sensitiveness may be detected by the P.-K. method of passive transfer, using a normal subject who gives negative skin reactions. Even this method occasionally fails to prove the point and the clinical history will have to be the guide as to whether specific treatment should be given.

TREATMENT. In the average case treatment is started with 0.1 ml. of the 1 : 10,000 dilution (or 1 : 1000 dilution, if the intracutaneous test to this concentration is slight), and the dose is increased gradually to 0.9 ml. giving six graded doses of the 1 : 10,000 and 1 : 1000 dilutions, eight doses from the 1 : 100 dilution, and nine from the 1 : 10 dilution. Treatment is started 8 to 10 weeks before the beginning of the season, and injections are given three times a week. If less time is available before the season starts, the doses are given more frequently. Then injections of 0.9 ml. of the 1 : 10 dilution are continued at five-day intervals throughout the season. Some workers advise attaining a maximum dose much higher than this. Many commercial treatment sets, which include only about half this number of injections and often are not continued throughout the season, are entirely inadequate in many cases.

The scheme of dosage must be altered to suit the needs of the individual patient, reducing the dose, and subsequently increasing it more gradually, if general reactions occur. The exact dose, however, cannot be gauged by the amount of local reaction to the preceding injection. Neither a local nor a constitutional reaction is necessary for successful treatment. Injections should be subcutaneous and great care taken to prevent any of the extract entering a vein.

No injection for testing or for treatment should be given unless adrenalin solution is at hand, and the patient should be under observation for at least a half hour in case an alarming general reaction should occur. Especial care should be taken at the onset of the season, since general reactions are more frequent at this time, and when passing from the weaker to the stronger extracts, as from 1 : 100 dilution to 1 : 10 dilution, etc. The symptoms resemble those occurring after the administration of horse serum in sensitive individuals, and must be treated in the same way.

Usually treatment is stopped at the end of the season, but somewhat better results are obtained if 0.1 to 0.2 ml. of 1 : 10 dilution is administered at intervals of two to four weeks throughout the year. If treatment is not begun until the season has started, benefit can often be obtained by giving daily injections of 0.1 ml. to 0.2 ml. of 1 : 1000 dilution, making no effort to increase the dose. Gay has found that ordinary air-conditioning methods effectively remove pollen from the air. Symptoms are usually relieved within a half hour after the affected individual enters an air-conditioned room, but often recur quickly after he or she leaves it. Some patients obtain more prolonged relief.

Asthma and Other Respiratory Allergies. These may depend upon the inhalation of other protein substances. Horse dander, the hair of various animals, feathers, orris root (in cosmetics), spores of various fungi, and a large variety of other protein materials in the air and dust in minute amounts may cause respiratory symptoms. These substances may often be identified by skin tests as described below.

It is often difficult to discover the exciting cause of these conditions since there are innumerable proteins with which an individual may come in contact; and in these cases hypersensitiveness to an extract of dust from the environment may be demonstrated. These individuals may also be benefited greatly by desensitization with the specific protein, if it can be identified, or with dust extracts. These may be prepared by saturating a cupful of dust with Coca's solution, and filtering after a two-day extraction. The solution is then sterilized by passing through a Berkefeld filter. A scratch test is made with this extract. If the reaction is positive, one should dilute the extract and follow the procedure previously outlined for pollen extracts. If it is negative, as it usually is, an intracutaneous test with 0.025 ml. of a 1 : 10 dilution is given, and if this is negative, with 0.025 ml. of undiluted extract. Treatment is started with 0.1 ml. of the dilution which causes a mild local reaction, giving injections twice weekly, and gradually increasing the dose to 0.3 ml. of undiluted extract. Patients who are highly sensitive to pollens or animal dander which may be present in the dust may give violent reactions to the undiluted extract. In many cases long-standing asthma is complicated by bacterial infection, and associated with hypersensitiveness to the bacterial protein.

Food Idiosyncrasies. These may be manifested in many different ways: urticarial eruptions or eczema (particularly in children), angioneurotic edema, gastrointestinal disturbances which may be violent, asthma, convulsions in children, and, according to some observers, attacks of migraine or epileptic seizures in predisposed individuals. The possibility of sensitization by absorption of foreign protein from the gastrointestinal tract has been demonstrated experimentally in animals. In certain individuals, particularly small children, the degree of hypersensitiveness may

be extreme. A small fraction of a drop of milk may precipitate a violent and dangerous general reaction.

In some individuals showing these symptoms a careful history will give a hint as to the food concerned. In a certain number of the cases, it may be determined by skin tests. These may be carried out by intracutaneous injections of extracts prepared in a manner similar to that outlined for pollens and dust. More often "scratch tests" are employed. Material for these tests may be purchased in the form of dried powders or glycerin pastes (containing the proteins precipitated from aqueous extracts of the foods). Small superficial scratches, which just fail to draw blood, are made an inch apart on the flexor surface of the forearm, or thigh or back. A portion of the paste, or the powder moistened with a drop of N/10 sodium hydroxide, is rubbed into the scratch. A positive reaction is indicated by the appearance of a small wheal, distinctly larger than the controls, usually within 5 to 30 minutes. The advantages of this method are the simplicity of the technic, the relatively greater stability of the extracts, and the rarity of general reactions, although these may occur. It is the most practicable method for the physician doing only occasional tests. An outspoken local reaction indicates a high degree of sensitiveness, and is usually of clinical significance.

"Intracutaneous tests" are about 100 times as sensitive as the scratch tests, and detect slight degrees of sensitiveness which are missed by the scratch method. However, intracutaneous tests frequently give slight or moderate reactions which are of no clinical significance. Greater discrimination is needed in their interpretation, and much greater caution to avoid general reactions. The wheals are much larger than those usually obtained by the scratch method.

Among the foods to which hypersensitiveness is most frequently encountered may be mentioned cow's milk, egg, wheat, sea foods, certain fruits (tomato, orange, and strawberry), nuts, and the commoner meats. Multiple sensitization is common, and an adequate examination requires a large number of tests. The reactivity of the skin varies, and repeated tests should be tried with suspected foods if necessary. If the skin is hyperreactive and an excessive number of positive reactions are obtained, no conclusions can be drawn. Tests should be made by passive transfer if possible.

In a considerable number of patients with food allergy, cutaneous reactions are negative. Such cases can sometimes be detected by the Prausnitz-Küstner technic, but often only by the use of elimination diets.

In many cases of chronic urticaria, angioneurotic edema, or eczema in adults the cause is not food allergy. Most such cases appear to be associated with nervous tension and vasomotor instability, or chronic focal infections.

TREATMENT. Treatment of patients with food idiosyncrasies consists in avoiding the food in question if practicable. Elimination from the diet must be complete. If difficult or impracticable as in the case of wheat or milk, desensitization may often be accomplished by giving a series of injections of extracts of the food, or more simply, by its administration by mouth. The food is completely eliminated from the diet, and increasing, measured doses are eaten daily, starting with minute amounts which give no symptoms. It must be taken continuously or sensitiveness may recur.

CONTACT DERMATITIS. This is often due to local allergic reactions to substances coming into direct contact with the skin, such as fabrics, dyes, furs, cosmetics, leaves of plants, drugs, chemicals, synthetic plastics, and dust. In such cases the patch tests of Bloch are much more useful than ordinary cutaneous tests.

A portion of the material is placed in the center of a piece of white blotting paper ⅛-inch square, or if the substance is liquid a bit of cotton or blotting paper is moistened

with it. The cotton is applied to a normal, hairless portion of the skin, covered with a piece of waterproof fabric, cellophane, or waxed paper, and fastened in place with adhesive tape. The material is applied in the same concentration as that in which it comes in contact with the skin naturally. The patch is left in place for 24 hours to 4 days unless discomfort occurs. A positive reaction reproduces the lesion with the appearance of papules or vesicles, usually within 24 or 48 hours, but occasionally only after four to seven days. A normal control must show no reaction. In the case of organic substances this appears to be due to a reaction to oils rather than to protein. If the offending oil cannot be avoided, purified preparations of these allergenic oils are now available for desensitization.

Drug Idiosyncrasies. Certain drugs may cause the appearance of allergic manifestations, especially after prolonged administration—quinine, salicylates, aminopyrine, morphine, luminal, phenolphthalein, arsenic preparations, etc. It is not clear how these nonprotein substances can elicit such reactions.

Zinsser believes that sensitization is due to an antigen formed in the body by the union of the chemical substance with body protein. This combination of drug and protein then sensitizes like a foreign protein. The drug portion of the combination corresponds to a haptene, and allergic reactions are brought about by its administration, just as the injection of other haptenes (for instance the carbohydrate of the pneumococcus) may cause anaphylactic shock in animals sensitized with the whole antigen. Skin tests rarely detect this form of hypersensitiveness.

Bacterial Allergy. An allergic condition, analogous to that observed in hay fever, may arise in an individual with a chronic or repeated infections, and a skin reaction may be obtained with the homologous bacterial protein. The best known example is that of the tuberculin reaction which is discussed in the section on the tubercle bacillus.

The mechanism of this form of allergy and its relationship to immunity are still not clear. Hypersensitiveness to bacterial bodies differs in its manifestations from that to serum and other proteins in solution, including bacterial proteins. In the latter ("anaphylactic" or "Arthus" type), the reaction, local or general, is immediate, and it is accompanied by spasm of smooth muscle. There are antibodies in the serum, and hypersensitiveness can be transferred passively to normal animals. In the cases of bacterial hypersensitiveness (or the "tuberculin type") the reaction is delayed for several hours, it is not accompanied by smooth-muscle spasm, the hypersensitiveness cannot be transferred passively to normal animals, and no antibodies can be demonstrated in the blood, although it seems probable that they are present in the body cells. Guinea pigs may be sensitized with tuberculoprotein as with other foreign protein so that typical anaphylactic reactions will develop on a second injection, but these animals will not give a positive cutaneous reaction to the tuberculin test and they show no immunity. On the other hand, if they are injected with dead or living tubercle bacilli, both a true protein anaphylaxis and cutaneous sensitivity can be demonstrated together with some degree of immunity.

In many other infections allergic reactions to the causative organism have been demonstrated. Colon bacillus extracts frequently give a slight reaction in normal individuals.

Tests may be made by intracutaneous injections of 0.025 ml. of solutions of purified bacterial proteins, or of a vaccine diluted to contain about 100,000,000 organisms per ml. A positive reaction may sometimes be manifested by the prompt appearance of a small urticarial wheal, or as a rule after 12 to 24 hours by an indurated papule surrounded by an erythema.

The clinical conditions in which the demonstration of allergic reactions to bacteria have thus far proved to be of practical importance are chiefly chronic asthmatic bronchitis and chronic infectious arthritis. In the latter, skin tests may be made with various strains of hemolytic and nonhemolytic streptococci, and treatment with a vaccine prepared from a reacting strain has seemed to some observers to be of value. Bacteria isolated from the sputum of asthmatic bronchitis may be tested in the same way, and vaccine treatment instituted with the reacting species. In these cases it may be difficult to determine whether improvement is due to specific desensitization or to a nonspecific foreign-protein reaction.

Respiratory and cutaneous symptoms indistinguishable from those of allergic sensitiveness may be due simply to an unusual vasomotor response to heat and cold.

In the dermatomycoses, chronic secondary eczematous lesions (epidermatophytids) appear which are due to an allergic reaction to the fungus protein. The hypersensitiveness can be demonstrated by intracutaneous injections or patch tests of "trichophytin," an extract prepared from the fungus (*Trichophyton*). Patients infected with other species belonging to this family (*Gymnoascaceae*) also react to trichophytin. In the case of monilia infections a specific *Monilia* extract (oidiomycin) must be used. Desensitizing injections are often beneficial (Sulzberger and Wise, 1932).

The presence of echinococcus infection may be determined by intracutaneous injection of sterile fluid from a cyst. Positive reactions occur within a half hour. Intracutaneous tests have also been employed in the diagnosis of lymphogranuloma inguinale and chancroidal infections, and of trichinosis, filariasis, and infections with other animal parasites, and of brucellosis. These are discussed in Chapters 8 and 24.

Skin tests related to bacterial infection are based upon two mechanisms: specific hypersensitivity to bacterial protein and lack of immunity. In the first category fall such tests as the tuberculin and the brucellin. In the second group are the Dick test and the Schick test. Table 18 summarizes pertinent information for the more common cutaneous tests.

Preparation of Vaccines

Whereas vaccines for prophylaxis are of necessity made from stock cultures, the use of autogenous vaccines, made from cultures direct from the patient, are preferable for treatment. In this way possible antigenic differences between various strains are avoided, and the particular organism obtained is less likely to be dissociated than a strain which has been artificially cultivated for many generations. When mixed cultures are obtained, however, as in cultures from sputum, it is necessary to use judgment in the selection of the species used in preparing a vaccine. Various methods of determining the significance of an organism obtained by culture have been suggested. The agglutination of a strain by the patient's serum, or a positive skin reaction on intradermal injection of the vaccine offers presumptive evidence that the organism is concerned in the infection. Thus, if a particular streptococcus isolated from the tonsils in a case of infectious arthritis gives a definite reaction on intradermal injection whereas other strains of streptococci do not, one may conclude that a vaccine from that strain is more likely to be beneficial than a stock vaccine. On the other hand, practically every mouth harbors streptococci of different kinds which are essentially saprophytic, and a vaccine made

Table 18

INTRACUTANEOUS DIAGNOSTIC TESTS

Name of Test	*Material*	*Administration*	*Recording*	*Remarks*
Schick test (for determining susceptibility to diphtheria)	Diluted diphtheria toxin; control is heated toxin	0.1 ml. intradermally; same amount of control on opposite arm	Read at end of 48 hours and record as positive or negative. A positive reaction shows edema and usually scaling for 7 days	The control rules out sensitivity to bacterial protein. In general a false reaction comes on earlier, fades faster, and leaves less pigmentation than a positive Schick reaction
Dick test (for determining susceptibility to scarlet fever)	Diluted erythrogenic toxin; no control needed	0.1 ml. intradermally	Read between 18 to 24 hours. Positive reaction is indicated by an erythema over 10 mm. in diameter. Record as positive or negative	Dick test reflects only presence or absence of antitoxin to the erythrogenic fraction. It has no significance as a measure of immunity to other streptococcal infections
Frei test (for determination of allergy to lymphogranuloma inguinale	Chick-embryo culture of the virus of lymphogranuloma inguinale	0.1 ml. intradermally with a similar amount of control on opposite arm	48 to 72 hours for first reading with subsequent check on 4th and 7th day; record presence of a papule greater than 6 mm. and without significant reaction of control as positive	This test is supposedly reliable in 90% of cases. A positive reaction may reflect past disease rather than present complaint. About 3 wk. must elapse between development of infection and appearance of a positive reaction
Tuberculin test (for determination of allergy to the tubercle bacillus)	Either PPD or old tuberculin	0.1 ml. intradermally. If PPD is desired use 1st dilution (.0002 mg./ml.) If O.T. use 1 : 10,000	Read in 36 to 48 hours. Record amount of redness and swelling by diameter in mm. This measures 10 mm. or more in a clear-cut reaction	When results are not clear-cut it is often advisable to use a dilution of greater strength and repeat the test. If marked sensitiveness is suspected, start with $\frac{1}{100}$ of these quantities
Ducrey test (for determination of sensitivity to *H. ducreyi*)	A suspension of the specific organisms	0.1 ml. intradermally. No control is necessary	A positive reaction appears in 48 hours and is manifested as an area of induration in excess of 7 mm. Record as positive or negative	Too little is known of this test for didactic appraisal. An attempt to isolate the organism from local lesion or lymph node should be made
Brucellin test (for demonstration of allergy to members of Brucella)	An extract of Brucella prepared by Michigan State College	0.1 ml. intradermally. No control is used	In 24 to 48 hours inspect for erythema or induration. An area of edema of over 20 mm. is recorded as a positive reaction	About 15% of people show positive reaction to the skin test so that the significance of the reaction in relation to symptoms must be carefully weighed
Trichinella test (for determination of sensitivity to trichina protein)	An extract of the worms	0.01 ml. intradermally, with a saline control	A positive reaction usually appears as a wheal with pseudopods within 20 minutes. Record measurements and presence or absence of pseudopods	There is evidence that some individuals may display a delayed type of reaction and reactions immediately negative should be checked in 24 hours. This test is believed to have about 95% specificity
Echinococcus (for determination of sensitivity to echinococcus [illegible])	An extract prepared from hydatid fluid by the N.I.H.	0.01 ml. intradermally, with saline control	A positive reaction is manifest by wheal and pseudopods within 20 minutes. Record measurements of wheal and presence or absence of pseudopods	Some individuals may show a delayed reaction and all negative reactions should be checked at the end of 24 hours

from them might be less desirable than a stock vaccine to which hypersensitiveness could be demonstrated.

The value of vaccine therapy in various conditions is discussed elsewhere. In evaluating the results of treatment in general, however, it is probable that desensitization and a nonspecific foreign-protein reaction play a part as well as specific antibody formation.

Cultures for vaccines may be made on appropriate solid media. Plain agar is preferable for the staphylococcus and the typhoid-colon group. Blood or serum agar or other special media are necessary for some organisms. Several slants are inoculated and incubated the minimum length of time necessary to obtain a good growth. The growth on each slant is then emulsified in 2 or 3 ml. of sterile salt solution by means of a platinum loop or small cotton swab. Each suspension is poured into a sterile test tube. If any fragments of the medium are present they must be removed by a short centrifugation. When blood agar is used, the suspension is sometimes so discolored by hemoglobin that it is necessary to sediment the bacteria by thorough centrifugation and resuspend them in fresh salt solution. If the suspension is in clumps, the tube may be sealed and shaken vigorously for 10 to 15 minutes, preferably in a shaking machine. If necessary, large clumps may be broken up with a sterile glass rod. In some cases relatively little shaking is required, whereas streptococcus vaccines may require a great deal of breaking up, and may need to be filtered through a little sterile cotton to remove the remaining aggregations.

As a routine procedure, for most organisms cultures are made in basic broth media. Several tubes are inoculated with the organism or organisms from which the vaccine is to be prepared. After 18 to 24 hours of incubation the cultures are centrifuged and the sediments are suspended in about 10 ml. of the supernatant broth to give a suspension four times heavier than that desired. If the finished vaccine is to contain 1 billion organisms per ml. the suspension at this point should, for example, contain 4 billion organisms per ml. If the finished vaccine is to contain 500 million organisms per ml. then the suspension should contain 2 billion organisms per ml. If the McFarland nephelometer scale is used for the adjustment of the suspension, the tube corresponding to a suspension of 2 billion organisms per ml. may conveniently be used as a standard. If a heavier suspension, like one containing 4 billion organisms per ml. is desired, one should dilute a small measured portion of the suspension with a measured amount of supernatant broth to the turbidity of the 2 billion standard and then dilute the rest of the suspension with the supernatant broth to give only one-half as much dilution.

The organisms may be killed by heat or by a chemical. In killing by heat the suspension is usually exposed to a temperature of 60° C. for one hour. This treatment will usually kill all vegetative forms (not spore-containing) of pathogenic bacteria. Thymol may be conveniently used by adding 0.1 ml. of an alcoholic (95 per cent ethyl alcohol) 5 per cent solution of thymol for every 5 ml. of the final volume of the vaccine. Thus, if the suspension prepared above amounts to 10 ml. the final volume of the vaccine should be 40 ml., and 0.8 ml. of the thymol solution should be added to the suspension. The latter is allowed to stand in the refrigerator overnight and is then diluted with sterile 0.85 per cent saline. In the example presented above, 29.2 ml. saline are added to provide an additional but weaker suspension.

The vaccine should be cultured for sterility by inoculating portions into fluid thioglycollate medium or a semisolid agar. Seven days of incubation are required before the vaccine may be designated "sterile."

The concentrated suspension is then standardized to determine the number of bacteria present in a given volume. For great accuracy this should be done before heating, since this undoubtedly results in some autolysis, but when virulent organisms are employed it is safer to kill the vaccine first. Several methods have been devised for standardization. These will be discussed presently.

After standardization phenol is added to a concentration of 0.5 per cent (or trikresol 0.25 per cent) of the total volume for preservation, if the vaccine was killed by heat. Vaccines are best kept in bottles containing a few glass beads for shaking, and capped with a rubber stopper with a thin top through which a needle may be plunged to remove each dose.

For treatment, staphylococcus and brucella vaccines are prepared in a concentration of 1 billion per ml. For most of the other vaccines a concentration of 100 million is preferable. Treatment is begun with one-tenth or one-twentieth of this amount subcutaneously. Subsequent injections are given at three- to seven-day intervals, gradually increasing the dose to 10 times the initial dose. In general the doses are so adjusted as to avoid any appreciable local or constitutional reaction.

When mixed vaccines are used, pure cultures of each organism are obtained, and separate vaccines are prepared and standardized. They are then combined in appropriate concentrations.

Vaccines have been prepared by various methods of autolysis and digestion. In general such preparations are antigenic unless the autolysis is carried too far, but it is questionable whether they have any advantage over other types.

Standardization of Vaccines: Wright's Method. In a capillary pipet with a mark about ½ inch from the tip, 1 volume of vaccine is drawn up, then a small air bubble, and then blood from the finger tip to the same mark. The contents of the pipet are mixed quickly; a smear is made on a slide and stained. The red cells and bacteria are counted in several areas. The number of bacteria per ml. $=$ ratio $\frac{\text{Bacteria counted}}{\text{Red cells counted}} \times 5{,}000{,}000 \times 1000.$

Hemacytometer Method. In this method a counting chamber is used, preferably the special Petroff bacterial counting chamber with a depth of 0.02 mm. However, an ordinary blood-counting chamber may be used with a sufficiently thin cover to enable one to focus on the bacteria clearly. The vaccine is drawn up to the 0.5-mark with either the red- or the white-cell pipet according to the concentration of the suspension, and then diluted with freshly filtered weak methylene blue or 0.05 per cent dahlia in 1 per cent formalin to the 11 (or 101) mark. Callison recommends the following diluting fluid, which promotes rapid sedimentation of the organisms. Hydrochloric acid 2 ml., bichloride of mercury (0.2 per cent) 100 ml., and sufficient 1 per cent aqueous solution of acid fuchsin to color a deep cherry red. This should be freshly filtered before using. The pipet is thoroughly shaken and the mixture is run into the counting chamber. The preparation must be allowed to stand at least one-half hour before counting to allow the bacteria to settle. By direct counting of the squares the number of organisms per ml. of the suspension can be calculated and dilutions made accordingly.

Nephelometric Method of McFarland. This is the simplest method, and is sufficiently accurate for most clinical purposes. If greater accuracy is necessary the results may be checked by direct counting. This method consists in comparing the opacity of the vaccine with a series of 10 standard tubes containing varying amounts of barium sulfate in suspension. These tubes may be prepared as follows: To a series of 10 tubes of uniform diameter increasing quantities of 1 per cent C.P. barium chloride solution are added, starting with 0.1 ml. in the first tube, and increasing the quantity by 0.1 ml. in each succeeding tube. Then to each tube enough 1 per cent C.P. sulfuric acid is added to bring the total volume to 10 ml. When sealed these may be kept for months, but should be renewed at least once a year. Another tube of the same diameter is kept sterilized for holding the vaccine for comparison. If vaccines are made direct from broth cultures without resuspending in saline, the barium sulfate standards must be backed with a tube of sterile broth.

In such tubes the density of the different suspensions will correspond approximately, in the case of most of the ordinary bacteria, to the following number of bacteria per ml.:

No.	*Number of Bacteria*	*No.*	*Number of Bacteria*
1	300,000,000	6	1,800,000,000
2	600,000,000	7	2,100,000,000
3	900,000,000	8	2,400,000,000
4	1,200,000,000	9	2,700,000,000
5	1,500,000,000	10	3,000,000,000

A bacterial standard containing 1 billion organisms per ml. can be prepared by adding 4 ml. sterile saline to 8 ml. triple typhoid vaccine (1500 million per ml.).

Put 1 ml. of the vaccine in a sterile tube (of the same diameter as the standard tubes) and dilute with a measured amount of sterile salt solution until it matches the density of one of the tubes. The tubes must be well shaken. Calculation: number of bacteria per ml. = number corresponding to the tube matched times the dilution. For example, if 1 ml. of vaccine is diluted to 5 ml. to match tube No. 3 it contains 900,000,000 × 5, or 4,500,000,000 organisms per ml. To make from this suspension 30 ml. of vaccine containing 500,000,000 per ml., the number of ml. of vaccine to be diluted to 30 will be

$$\frac{30 \times 500{,}000{,}000}{4{,}500{,}000{,}000} = 3.33 \text{ ml.}$$

For greater accuracy several of the prepared tubes may be checked with vaccines of known concentration. The figures given above apply to organisms about the size of the staphylococcus, streptococcus, gonococcus, and colon bacillus. Vaccines of the influenza bacillus contain about three times as many organisms as these standards would indicate.

Therapeutic Use of Vaccines. For the most part vaccines are not indicated in acute infections. In certain chronic or subacute infections they may be beneficial. They have been used particularly in furunculosis, sinus infections, otitis media, bronchitis (especially when associated with asthma), pyelitis and cystitis, chronic gonorrheal infections, infectious arthritis, and other low-grade, chronic infectious processes.

The prophylactic use of typhoid vaccine, tubercle bacillus preparations, and other vaccines is discussed under their respective headings.

Typhoid vaccine has been given intravenously for the production of protein shock and fever in the treatment of chronic arthritis and many other diseases, including syphilis, especially paresis and Wassermann-fast cases. Doses should be small, not over 25 or 50 million for the first dose, and should not be increased beyond 300 million.

Serodiagnostic Tests for Syphilis*

There are two types of serodiagnostic tests for syphilis, namely, the complement-fixation and the precipitation tests. The various precipitation tests are also spoken of as flocculation tests.

Wassermann, Neisser, and Bruck, in 1906, were the first to utilize the phenomenon of Bordet and Gengou in the serodiagnosis of syphilis. At first the reaction as applied to syphilis was thought to be a true antigen-antibody reaction. This was disproved when it was shown that alcoholic extracts of normal tissues were more sensitive and specific than aqueous extracts of syphilitic tissues. The addition of cholesterol to the alcoholic extract containing the lipoidal fraction of the tissue

*This section, "Serodiagnostic Tests for Syphilis," (pp. 305–336) was written by Lieutenant Genevieve Stout H(W) USNR, Naval Medical School, NNMC.

improves the antigenic value and forms the basis of the antigens used in the various diagnostic tests for syphilis. The two best known and widely used complement-fixation tests are the Kolmer and the Eagle.

The phenomenon of precipitation with syphilitic sera was described in 1907 by Michaelis. The earlier tests were not sufficiently sensitive or specific to be of diagnostic value. In 1921 Kahn began to study the precipitation phenomenon with syphilitic sera and developed a rapid test which was so superior to the slow precipitation technics that it attained wide use throughout the world. Since then a number of other flocculation tests have been developed which are, as a rule, known by the names of their respective authors. The best recognized of proved value are those of Eagle, Hinton, Kline, and Mazzini.

In the pages following, the details of the technics for the Kahn precipitation test and the Kolmer complement-fixation test are described.

Kahn Test Procedures

Apparatus. The use of standard apparatus in the performance of the various procedures of the Kahn tests is absolutely essential for correct results.

1. Kahn test tubes for the performance of the tests (with serum and spinal fluid) are 75 mm. in length and 10 mm. in inside diameter, and are made of Pyrex glass.
2. Antigen-suspension vials (with straight wall and flat bottom) for preparing Kahn antigen suspensions are 55 mm. in length and 15 mm. in inside diameter.
3. Pipets: The following pipets are needed:
   10.0-ml. graduated to 0.1 ml.
   1.0-ml. graduated to 0.01 ml.
   0.25-ml. graduated to 0.0125 ml. (for pipetting antigen suspension)
   0.2-ml. graduated to 0.001 ml.
4. Blood-specimen test tubes. The recommended size of test tubes for the collection of blood specimens is 100 mm. × 15 mm., Pyrex.
5. Serum test tubes. The recommended size of test tubes for holding the individual serums to be tested is 100 mm. × 13 mm., Pyrex.
6. Kahn test-tube rack made of suitable material (preferably rubberized sheet copper) is 3 inches wide, 11½ inches long, 2¾ inches high, and consists of three shelves, the upper and middle ones containing three rows of holes each approximately ½ inch in diameter. The center row of holes is offset ½ inch to the left.
7. Kahn shaking apparatus. This apparatus has a speed of 275 to 285 oscillations per minute, with a stroke of 1½ inches.
8. Water bath must be of such type as to maintain a temperature of 56° C.
9. Centrifuges and metal centrifuge tubes.
10. All glassware must be scrupulously clean. Tubes and vials are rinsed thoroughly until absolutely clean and free of blood, serum, etc., and they are then boiled for 15 minutes in soft water containing a very mild soap such as Ivory Flakes. One must be absolutely certain that the soap is in solution before adding to the tubes. Then the tubes and vials are rinsed thoroughly in soft water and placed in dichromate cleaning solution overnight (12 to 24 hours), after which they are rinsed in soft water until free of all cleaning solution (three to four rinsings) and then are rinsed twice in distilled water. They are then inverted in wire baskets and dried thoroughly in an oven.

Pipets are rinsed thoroughly (immediately after use) in cool tap water to force out

of the bores all serum, etc. They should then be left immersed in tap water in a museum jar until they can be put into the cleaning solution. They are placed in cleaning solution overnight, after which they are rinsed thoroughly in soft water and twice in distilled water. The pipets are then placed in a slanted position in a wire basket and dried thoroughly in an oven. If cleaning solution is not employed, a layer of organic material will accumulate on the glassware. Automatic Pipet Washers are an aid in the laboratory having a big load and lacking in personnel. Copper pipet boxes are also recommended, if available, for their convenience and for their protection to the pipets.

Reagents. Antigen. The antigen for the Kahn test is a specially prepared alcoholic extract (cholesterolized) of beef heart from which the ether-soluble elements have been partially removed. Each lot of antigen must be standardized to render it comparable in sensitivity and specificity to standard Kahn antigen. The following steps are employed: (1) Determination of titer; the minimum amount of 0.9 per cent salt solution to be added to 1 ml. antigen producing a suspension of aggregates which undergoes complete visible dispersion upon addition of further salt solution. (2) Determination of sensitivity and specificity; antigen is tested at its titer with syphilitic and nonsyphilitic sera, employing simultaneously standard antigen as a control. (3) Correction of antigen to standard requirements. It is distinctly advantageous to obtain Kahn antigen from well-controlled central laboratories where it is prepared in large amounts. For details regarding the preparation and standardization of Kahn antigen, see *Technique of the Standard Kahn Test and of Special Kahn Procedures,* by R. L. Kahn, University of Michigan Press, Ann Arbor, Michigan (1944).

Standard Kahn antigen is stable and will maintain the same titer and remain uniform in sensitivity and specificity for many years provided: (*a*) it is stored in chemically clean dry bottles and tightly stoppered with screw caps lined with tin foil or vinylite, and (*b*) is kept at room temperature in a dark place.

Saline Solution. The saline solution consists of 0.9 per cent sodium chloride (C.P.) in distilled water. The sodium chloride should be dry before weighing. Kahn saline solution should be prepared every few days and always filtered before using.

Serum. The blood specimen is centrifuged to separate the serum from the clot. The clear supernatant serum is either poured off into a clear tube, or it is pipetted off with a serum-transfer, bulb-capillary pipet. The same transfer pipet must never be used for more than one specimen until it is recleaned. It is essential that the serum be entirely free from cells or other particles, since they may give the impression of a precipitate in the completed test.

The results of the Kahn test are not affected if the sera show moderate hemolysis or bile, but if markedly hemolyzed or decomposed because of bacterial contamination they are not fit for serologic examination.

The clear serum should be heated (inactivated) for 30 minutes in a water bath at a temperature of 56° C. and then examined for particles. If present, the specimen must be recentrifuged. Sera should be tested as soon as possible after being heated. For uniformity, it is well to begin the performance of the tests within 10 minutes after the sera have been removed from the water bath. Sera that have been heated 2 to 24 hours previously should be reheated for 10 minutes at 56° C. when they are to be re-examined; if after 24 hours, they should be reheated for 15 minutes.

Standard (Diagnostic) Test with Serum. The standard test is a three-tube test, each tube containing a different proportion of serum to antigen suspension. Optimum precipitation is obtained when the concentration of antigen and antibody (reagin) approximate one another. Hence, a relatively large, moderate, and small quantity of antigen suspension is employed with each serum, since the serum may contain a large, moderate, or small amount of antibody. Then again, the use of three proportions of serum to antigen

suspension makes it possible to obtain highly sensitive reactions with standard antigen which is of moderate sensitivity, but of high specificity.

The following outline presents the general plan of the standard Kahn reaction giving four different ranges in precipitation:

	Tube 1	*Tube 2*	*Tube 3*
Serum-antigen suspension ratio	3 : 1	6 : 1	12 : 1
Antigen suspension, ml.	0.05	0.025	0.0125
Serum, ml.	0.15	0.15	0.15
Illustrative Types of Precipitation Reactions:			
Negative in the three proportions	—	—	—
Positive in the three proportions	++++	++++	++++
Positive only with the small amounts of antigen suspension	—	++	++++
Positive only with the larger amounts of antigen suspension	++++	++	—

PROCEDURE: 1. PREPARATION OF STANDARD ANTIGEN SUSPENSION. This suspension is prepared shortly before the sera are taken from 56° C. water bath. Antigen is mixed with salt solution according to the required titer. Thus, if the titer is 1 ml. antigen plus 1.2 ml. physiological salt solution, the antigen is mixed as follows: (*a*) 1.2 ml. salt solution is measured (with a 1-ml. or 2-ml. pipet) into a chemically clean and dry standard antigen-suspension vial; (*b*) 1 ml. antigen is measured (with a chemically clean and dry 1-ml. pipet) into a similar vial; (*c*) the salt solution is poured into the antigen, and as rapidly as possible (without waiting to drain the vial) the mixture is poured back and forth a total of 12 times to ensure thorough mixing; (*d*) the antigen suspension is allowed to stand for 10 minutes before using. The suspension is not to be used after standing over 30 minutes from the time of mixing. A previously prepared antigen suspension is not to be mixed with a newly prepared suspension. More than 1 ml. of antigen may be mixed with a proportionately larger amount of salt solution. Thus, in case of an antigen with the above titer, 2 ml. may be mixed with a 2.4-ml. salt solution and 2.5 ml. with a 3.0-ml. salt solution. Amounts of antigen less than 1 ml. or more than 3 ml. for the preparation of an antigen suspension should not be used.

2. MEASURING ANTIGEN SUSPENSION. After standing 10 minutes, the antigen suspensio is shaken well, and 0.05-, 0.025-, and 0.0125-ml. amounts are measured for each serum delivering the suspension to the bottom of the tubes. The standard rack capacity is 30 tubes; 0.05-ml. amounts are measured into the tubes of the first row, 0.025-ml. amounts in the tubes of the second row, and 0.0125-ml. amounts in the tubes of the third row.

3. MEASURING SERUM. The serum is added as soon as possible after the antigen suspension has been pipetted to avoid undue evaporation of the suspension. It is of the utmost importance to use a clean, dry pipet for each serum. Each serum, in 0.15-ml. amounts, is added to the 0.05-, 0.025-, and 0.0125-ml. amounts of antigen suspension, and the rack of tubes is shaken vigorously for 10 seconds to ensure thorough mixing of the ingredients. The serum-antigen mixtures should stand for about five to seven minutes (preferably not less than three minutes and not more than 10 minutes) at room temperature before the mechanical shaking for three minutes (see "Shaking" below).

4. CONTROLS. Before measuring the antigen suspension and serum for the regular tes three control tests are carried out, one with a positive serum, one with a negative serum, and one employing salt solution instead of serum. The antigen suspension for these con-

trols is pipetted immediately after it has stood for 10 minutes. After pipetting the sera (and saline), the tubes are at once shaken for three minutes. This control set-up, completed in not more than five minutes, permits the reading of the results of the control tests before performing the regular tests and makes possible the detection of any error in the preparation of the antigen suspension.

5. SHAKING. The tubes are shaken in a standard shaking machine for three minutes. The machine has a speed of 275 to 285 oscillations per minute, with a stroke of 1½ inches.

6. ADDITION OF SALT SOLUTION. After the three-minute shaking period, 1-ml. amounts of salt solution are added to the tubes of the first row of the rack (containing the 0.05-ml. amounts of antigen suspension) and 0.5-ml. amounts of salt solution to the remaining tubes. The rack is shaken by hand sufficiently to mix the ingredients.

READING OF RESULTS. Results are read immediately after the addition of salt solution, and a check reading is made 15 minutes later, the racks remaining at room temperature during this interval. A permanent record is made of both readings. If the first reading is made in about five minutes after the addition of salt solution, the second reading should be made 10 minutes later. The final result is the average of the first and second reading. If it is impossible to read the results twice, then they should be read in from 5 to 10 minutes after the addition of salt solution.

Optimum reading conditions in each laboratory should be determined by trial, and workers should limit themselves to one method of reading.

When utilizing daylight for reading the results, it is well to have but one source of light coming from a single window immediately in front of the reader. It will be found satisfactory to shade the upper and lower portions of the window, narrowing the source of light to a section several feet in height. Light from any other windows near the reader should be dimmed by lowering the window shades. When holding the rack in front of the exposed section of the window, the definitely positive and the negative reactions may be readily differentiated without lifting the tubes from the rack. In case of weak reactions each tube should be examined individually, lifting it several inches above the eye level and slanting it until the fluid is spread into a thin layer. The precipitate will then become readily visible.

Those preferring magnification will find the microscope mirror helpful. The mirror is placed on a reading table (black top) with its concave surface upward. The tube, in slanted position, is held about 2 inches above the mirror and the image in the mirror is examined. Either daylight or artificial light (for example, the microscope lamp) may be employed. An adjustable fluorescent lamp is most satisfactory. One must make certain that the light is not too bright and that, when looking into the mirror, one does not see the reflection of the light bulb itself but only the magnified image of the Kahn reaction in the tube.

INTERPRETATION OF RESULTS. 1. The reaction in each of the three tubes of the test is read independently. A definite precipitate suspended in a clear medium is read four plus. Proportionately weaker reactions are read three, two, and one plus, and plus-minus or doubtful, respectively.

2. Strongly potent sera show complete or four-plus precipitation in each of the three tubes, but owing to the different amounts of antigen suspension employed the precipitates are unequal in bulk, being greatest in the first tube and least in the last tube.

3. Sera that are not strongly potent do not show complete precipitation in each of the three tubes. Such sera show most marked precipitation in the third tube because a small amount of antibody (reagin) reacts best with a small amount of antigen suspension. These sera generally show weak precipitation in the middle tube which contains a moderate amount of antigen suspension, and no precipitation in the first tube which contains a relatively large amount of the suspension.

4. Another type of precipitation reaction is met with occasionally, namely, one in

which precipitation is marked in the first tube and weak or negative in the second or third tube. In this instance, the serum generally is so markedly rich in antibody that it requires a relative excess of antigen suspension to give maximum precipitation. When a reaction of this type is encountered it is necessary to set up a supplementary test in which the amount of antigen suspension in relation to serum is increased beyond that employed in the standard test. A supplementary test is set up in which 2 : 1 and 1 : 1 proportions of serum to antigen suspension are used, thus:

SUPPLEMENTARY KAHN TESTS

	1st Tube	*2nd Tube*
Ratio	1 : 1	2 : 1
Antigen, ml.	.05	.05
Serum, ml.	.05	.1

The tubes are shaken three minutes. After shaking, 1.0 ml. saline is added to each tube. These two tubes, or at least tube 2, should show definite flocculation if the serum is strongly positive.

As an additional check on sera giving precipitation reactions in the first tube of the standard test and negative reactions in the remaining two tubes, a second supplementary test is made by setting up a partial quantitative test. Thus, the serum is diluted 1 : 5, 1 : 10, and 1 : 20 with salt solution and each dilution is examined with antigen suspension in a proportion of 15 : 1, in accordance with the following outline:

	Tube 1	*Tube 2*	*Tube 3*
Antigen suspension, ml.	0.01	0.01	0.01
Diluted serum, ml.	0.15 of 1 : 5	0.15 of 1 : 10	0.15 of 1 : 20
The tubes are shaken three minutes.			
Then the salt solution is added, ml.	0.5	0.5	0.5

If one or more tubes should show definite flocculation, the reaction of the serum can be considered positive.

If the supplementary examinations do not show positive reactions, then the three-tube test, which shows marked precipitation in the first tube and negative precipitation in the remaining two tubes, must be considered as giving a weak or doubtful reaction.

Rarely one finds that the three-tube tests show a borderline precipitate in each of the three tubes, such as ±, ±, ±; +, +, +; or perhaps ++, ++, ++. After ascertaining that these borderline reactions are not due to serum particles, the two supplementary tests described above are carried out. If these two tests show definite flocculation, the reactions are reported as positive. If reaction to the supplementary tests is also borderline or questionable, the chances are that it is nonspecific, owing to conditions other than syphilis.

REPORTING RESULTS. The final result to be reported to the physician is determined by adding the plus signs in the six readings and dividing by six (± reading is disregarded). The first reading is the one made immediately after the addition of saline, and the second reading is the one made after the racks have stood 15 minutes at room temperature. The following tables illustrate the method of reporting.

Table 19

TYPES OF REACTIONS REPORTED "POSITIVE (++++)"

Serum Number	First Reading			Second Reading		
	Tube 1	Tube 2	Tube 3	Tube 1	Tube 2	Tube 3
1	++++	++++	++++	++++	++++	++++
2	++++	++++	++++	++	++++	++++
3	+++	++++	++++	+++	++++	++++
4	++++	++++	+++	++++	++++	+++
5	++++	++++	++	++++	++++	++
6	++++	++++	+	++++	++++	+
7	++++	++++	—	++++	++++	—

Experience has shown that sera reacting as in 4, 5, 6, and 7 (zone phenomena) are strongly positive; hence, they are reported as "positive (++++)."

Table 20

TYPES OF REACTIONS REPORTED "POSITIVE (++++)" PROVIDED SUPPLEMENTARY EXAMINATIONS 1 AND 2 ARE POSITIVE

Serum Number	First Reading			Second Reading		
	Tube 1	Tube 2	Tube 3	Tube 1	Tube 2	Tube 3
1	++++	+++	+++	+++	+++	+++
2	++++	+++	++	++++	+++	++
3	++++	+++	—	++++	+++	—
4	++++	+	—	++++	—	—
5	+++	+++	+++	+++	+++	+++
6	++	++	++	++	++	++
7	++	—	—	++	—	—
8	+	+	+	+	+	+
9	+	—	—	+	—	—
10	±	±	±	±	±	±
11	±	—	—	±	—	—

Table 21

TYPES OF REACTIONS REPORTED "POSITIVE (+++)"

Serum Number	First Reading			Second Reading		
	Tube 1	Tube 2	Tube 3	Tube 1	Tube 2	Tube 3
1	+++	++++	++++	++	++++	++++
2	++	++++	++++	—	+++	++++
3	±	++++	++++	—	++++	++++
4	+	++++	++++	±	+++	++++

Table 22

TYPES OF REACTIONS REPORTED "POSITIVE (++)"

Serum Number	First Reading			Second Reading		
	Tube 1	Tube 2	Tube 3	Tube 1	Tube 2	Tube 3
1	±	++++	++++	—	+++	++++
2	—	++	++++	—	+	++++
3	—	+++	+++	—	++	+++
4	±	+++	+++	—	+	+++

Table 23

TYPES OF REACTIONS REPORTED "DOUBTFUL (+)"

Serum Number	First Reading			Second Reading		
	Tube 1	Tube 2	Tube 3	Tube 1	Tube 2	Tube 3
1	—	+	++++	—	+	+++
2	—	—	+++	—	—	++
3	—	++	++	—	+	++
4	—	+	++	—	+	++

Table 24

TYPES OF REACTIONS REPORTED "DOUBTFUL (±)"

Serum Number	First Reading			Second Reading		
	Tube 1	Tube 2	Tube 3	Tube 1	Tube 2	Tube 3
1	—	±	++	—	±	++
2	—	—	++	—	—	++
3	—	+	++	—	—	+
4	—	+	++	—	+	+
5	—	—	++	—	—	+

Table 25

TYPES OF REACTIONS REPORTED "NEGATIVE (±)"

Serum Number	First Reading			Second Reading		
	Tube 1	Tube 2	Tube 3	Tube 1	Tube 2	Tube 3
1	—	—	++	—	—	—
2	—	—	++	—	—	±
3	—	+	+	—	—	+
4	—	+	+	—	±	+
5	—	+	+	—	+	+
6	—	±	+	—	±	+

Table 26

TYPES OF REACTIONS REPORTED "NEGATIVE"

Serum Number	*First Reading*			*Second Reading*		
	Tube 1	*Tube 2*	*Tube 3*	*Tube 1*	*Tube 2*	*Tube 3*
1	—	+	+	—	—	—
2	—	±	+	—	—	+
3	—	±	+	—	±	±
4	—	±	±	—	±	±
5	—	—	+	—	—	+
6	—	—	±	—	—	±

Quantitative Test with Serum. The standard Kahn test is only partially quantitative. A serum might give a ++++ reaction or a +++, ++, + or a doubtful (±) reaction, but two sera giving ++++ reactions might show marked variations in their potency. The extent of this difference may be readily determined by use of the quantitative test. Positive sera are first diluted, in series, with salt solution. Each serum dilution is then examined with standard antigen suspension. The highest dilution giving a positive precipitation reaction is the end point desired. The apparatus and reagents are the same as for the standard test.

DILUTION OF POSITIVE SERUM WITH SALT SOLUTION. Five Kahn tubes are set up. Into tube 1 is measured 0.3 ml. of the 0.9 per cent salt solution, and into each of tubes 2, 3, 4, and 6, 0.2 ml. of the salt solution. To tube 1 is now added 0.2 ml. of the serum to be tested with a 0.2-ml. pipet. The mixture of serum and salt solution is drawn up in the pipet and emptied by blowing out the contents into the tube. This is repeated three times to assure the proper mixing of serum and salt solution. With the same pipet, 0.2 ml. of the mixture is transferred to tube 2. Mixing and transferring are continued as before through tube 5, where the pipet is permitted to remain.

The accompanying table gives the scheme for preparing the serial dilutions of serum:

Table 27

SERIAL DILUTIONS OF SERUM—QUANTITATIVE KAHN TEST

Tube	*Solution 0.9 % NaCl*	*Substance (Serum and/or Mixture of Serum and Salt Solution) Added*	*Final Dilution*
1	0.3 ml.	0.2 ml. of undiluted serum	1 : 2.5
2	0.2 ml.	0.2 ml. of mixture from tube 1	1 : 5
3	0.2 ml.	0.2 ml. of mixture from tube 2	1 : 10
4	0.2 ml.	0.2 ml. of mixture from tube 3	1 : 20
5	0.2 ml.	0.2 ml. of mixture from tube 4	1 : 40

Note: The serum dilutions should not be allowed to stand longer than 30 minutes before their transfer to the antigen-suspension tubes. Preferably, the serum dilutions should be employed within 10 to 20 minutes after their preparation.

PERFORMANCE OF TEST. The antigen suspension is prepared in the usual manner by mixing Kahn standard antigen with 0.9 per cent salt solution, according to the antigen titer. After standing 10 minutes (but not longer than 30 minutes) the antigen suspension is pipetted in 0.01-ml. amounts into each of five Kahn test tubes, the suspension being deposited at the bottom of the tubes. With the pipet that has remained standing in the highest serum dilution (tube 5), 0.15 ml. is transferred into the corresponding tube 5 containing the antigen suspension. Then, with the same pipet, 0.15 ml. of serum-dilution

tube 4 is transferred to antigen-suspension tube 4. In the same way, all the serum dilutions are transferred to the antigen-suspension tubes, in series. The rack of tubes is shaken for three minutes in the mechanical shaker (275 to 285 oscillations per minute). To each tube is then added 0.5 ml. of 0.9 per cent sodium chloride solution, the rack is shaken for 10 seconds to assure proper mixing and the results are read.

Determination of Kahn Units. A definite precipitate (++++, +++, or ++) is recorded as positive, while a very weak (+ or ±) reaction is disregarded. If a serum gives a ++++, +++, or ++ Kahn reaction (in an undiluted state) and reaction is negative in the dilution series, the serum is considered for simplicity in calculation as containing 4 units, 3 units, or 2 units, respectively. The potency of any serum which is positive on dilution is determined according to the formula $S = 4D$, where S is the serum potency in terms of Kahn units and D is the highest dilution ratio giving a positive (++++, +++, or ++) reaction. Thus, if serum dilution 1 : 5 is positive and 1 : 10 and higher dilutions are negative, the serum contains 5×4 or 20 Kahn units. If serum dilution 1 : 10 is positive and 1 : 20 and higher dilutions are negative, the serum contains 40 units.

Reporting Results. Results are reported "4 Kahn units," "40 Kahn units," etc., as the case may be.

Highly Potent Sera. If a serum gives a positive precipitation reaction in a dilution of 1 : 40, still higher dilutions of serum (1 : 60, 1 : 80, 1 : 100, 1 : 150, and 1 : 200) are examined with antigen suspension until a positive reaction is no longer obtained. Higher dilutions of serum may be readily prepared in accordance with the following scheme:

1. A 1 : 10 dilution of serum with 0.9 per cent salt solution is prepared by mixing 0.1 ml. of undiluted serum with 0.9 ml. of 0.9 per cent salt solution.

2. With the 1 : 10 dilution as a base, dilutions of 1 : 60, 1 : 80, 1 : 100, 1 : 150, and 1 : 200 are prepared.

The following is an outline of the scheme:

Table 28

Serial Dilutions of Highly Potent Serum—Quantitative Kahn Test

Tube	*Solution 0.9 % NaCl*	*1 : 10 Dilution of Serum (0.1 ml. Serum to 0.9 ml. of 0.9% Salt Solution)*	*Final Dilution*
6	0.5 ml.	0.1 ml.	1 : 60
7	0.7 ml.	0.1 ml.	1 : 80
8	0.9 ml.	0.1 ml.	1 : 100
9	0.7 ml.	0.05 ml.	1 : 150
10	0.95 ml.	0.05 ml.	1 : 200

Standard (Diagnostic) Test with Spinal Fluid. In this test the spinal-fluid globulins are precipitated by means of ammonium sulfate and redissolved in an amount of physiological salt solution equivalent to one-tenth of the original spinal-fluid volume. The concentrated globulin solution thus obtained is then examined with standard antigen suspension.

Preparation of Concentrated Globulin Solution: 1. reagents. The reagents needed for the preparation of concentrated globulin solution are (*a*) spinal fluid, (*b*) physiological salt solution, and (*c*) a saturated solution of ammonium sulfate of the highest purity ("reagent" quality). C.P. ammonium sulfate is not of sufficient purity for this test. Triply distilled water is employed in making this solution.

2. procedure. (*a*) Spinal fluid is centrifuged to render it free from cells and foreign particles. (If blood is present or fluid is cloudy, it should be so noted on the report.)

(*b*) 1.5 ml. of the clear fluid is added to a standard Kahn test tube (7.5 by 1 cm.).

(*c*) To the same tube is added 1.5 ml. saturated solution of ammonium sulfate.

(*d*) The fluids are mixed by covering the mouth of the tube with the thumb (protected with rubber) and shaking tube back and forth vigorously. The mixture is placed in a 56° C. water bath for 15 minutes to hasten the precipitation of the globulins.

(*e*) The mixture is centrifuged at high speed (about 2000 r.p.m.) for 15 minutes to throw down the precipitated globulins completely. The supernatant fluid should be water clear.

(*f*) The supernatant fluid is removed as completely as possible with a finely drawn capillary pipet. An optional method for removing this fluid is to pour it off rapidly and invert the tube in a standard rack over clean filter paper, allowing the moisture that adheres to the tube to drain and be absorbed by the paper for a period of 10 minutes. This draining period is not necessary if the inside of the tube is wiped dry by means of filter paper. The filter paper is first wound around a glass rod or pencil and is then inserted into the tube, without touching the precipitate. Fresh filter paper is employed for wiping each tube.

(*g*) Salt solution (0.9 per cent) in the amount of 0.15 ml. is added to the globulin precipitate which is redissolved readily by gentle shaking. In adding this salt solution the point of the pipet is held close to the bottom of the tube to avoid washing down traces of ammonium sulfate that may adhere to the inner wall. In isolated instances, when an excess of globulin is present in the spinal fluid, the globulin precipitate may require somewhat more than 0.15 ml. saline for complete solution.

(*h*) This globulin solution should now be inspected for clarity and for freedom from particles; it is then ready to be examined.

Preparation of Antigen Suspension. Salt solution is mixed with antigen in the same manner as for the standard test with serum, according to the antigen titer required for spinal fluid. The antigen suspension is allowed to stand 10 minutes and must be used in the test within the next 20 minutes.

Measuring Antigen Suspension. With a 0.2-ml. pipet graduated to 0.001 ml., 0.01 ml. antigen suspension is measured to the bottom of a standard Kahn test tube.

Table 29

Spinal Fluid—Standard Test

	Tube 1	*Tube 2*
Ratio of concentrated globulin solution: antigen suspension	15 : 1	15 : 1
Antigen suspension, ml.	0.01	0.01
Concentrated globulin solution, ml.	0.15	0.15
Shake by hand for 10 seconds, then four minutes in mechanical shaker.		
Salt solution, ml.	0.5	0.5

Measuring Concentrated Globulin Solution. With another 0.2-ml. pipet, 0.15 ml. of the concentrated globulin solution is introduced into the antigen-suspension tube. The rack is shaken vigorously for 10 seconds to mix ingredients.

Controls. Positive and negative spinal fluid controls are included.

Shaking. After mixing the globulin solution with antigen suspension, the test tubes are shaken at the standard speed for four minutes.

Addition of Salt Solution. To the tubes is added 0.5 ml. physiological salt solution.

Reading Results. Four-plus, three-plus, and two-plus reactions are reported as positive; one-plus reactions are reported as doubtful and plus-minus and negative reactions are reported negative.

Quantitative Test with Spinal Fluid. The method consists of examining with standard antigen suspension either serial dilutions of spinal fluid with saline or serial dilutions of the globulin solution with saline, as follows:

1. Examine 0.15 ml. centrifuged spinal fluid with 0.01 ml. standard antigen suspension. If the reaction (after shaking for four minutes) is positive, then serial dilutions of the spinal fluid with saline are prepared and each dilution examined with antigen suspension. Generally, 1 : 15, 1 : 20, and 1 : 30 dilutions of the spinal fluid with saline are ample. Since, in the qualitative (standard) Kahn test with spinal fluid, a four-plus reaction is based on a globulin solution which is ten times as concentrated as the spinal fluid, it follows that when the spinal fluid itself gives a four-plus reaction, this reaction is ten times more potent than the reaction with globulin. Hence, when a spinal fluid without concentration gives a positive reaction, the number of Kahn units is 10 × 4, or 40, and if two parts (0.2 ml.) spinal fluid are diluted with one part (0.1 ml.) saline and this dilution gives a positive reaction, the number of Kahn units is 60, provided, of course, that higher dilutions of the spinal fluid give negative reactions.

2. If the spinal fluid gives a negative reaction with antigen suspension and reaction to the standard test with the globulin solution is positive, then a 1 : 5 dilution of the globulin solution with saline is prepared and examined with antigen suspension. If this 1 : 5 dilution gives a positive reaction the quantitative titer is 20 Kahn units; if it gives a negative reaction the titer is based on the results with the undiluted globulin solution.

Presumptive Kahn Test with Serum. The presumptive Kahn test is a one-tube procedure employing a sensitized antigen that renders the test more sensitive than the standard Kahn test. Presumptive Kahn antigen or sensitized antigen is prepared by adding sensitizing reagent in combination with cholesterolized alcohol to standard Kahn antigen. It is standardized so that it possesses a uniform degree of sensitivity as does standard Kahn antigen. (See illustrations on pp. 318, 319, and 320.)

The presumptive test is of value (*a*) as a technical check on the standard test, (*b*) in treated cases of syphilis when a highly sensitive method may be desired, (*c*) as an additional criterion in establishing the absence of syphilis, and (*d*) as a "screen" test. The test is being used widely as a screen test because it conserves both time and material. All specimens examined with the presumptive Kahn test and found to be negative may be reported without further examination with an extremely small chance of error. Every serum showing any reaction whatsoever by the Kahn presumptive test must be checked by the Kahn standard (diagnostic) test.

Procedure. Presumptive antigen suspension is prepared as follows: (1) Into an antigen-suspension vial is measured 1 ml. sensitized antigen. (2) The amount of physiological salt solution, indicated by the titer of the antigen, is measured into a similar vial. (3) The salt solution is poured into the antigen vial and the mixture is poured, as rapidly as possible, back and forth twelve times. (4) The antigen suspension is allowed to stand at room temperature for 10 minutes before using, and is used in the test within the next twenty minutes.

Controls. A standard antigen suspension is prepared, according to previous directions, at the same time as the presumptive antigen suspension. Standard and presumptive control tests are set up with positive and negative sera and with salt solution. Only the 6 : 1 ratio of serum to antigen suspension is employed with positive and negative sera, and only the 3 : 1 and 6 : 1 ratios are employed with the salt solution. Immediately following completion of the test the results are read with the following aspects in mind: (1) the positive control should contain a precipitate; (2) the negative serum and saline controls should be free from particles and flakes; and (3) the general appearance of the negative serum and saline controls with standard and sensitized antigen should be the same. If these controls with sensitized antigen are cloudier than those with standard antigen, the amount of salt solution for the proper titer of the sensitized antigen should be slightly increased, generally by 0.05 ml. If the negative serum and saline controls are clearer than those of standard antigen, then the amount of salt solution in the sensitized antigen titer should be slightly decreased, generally by 0.05 ml.

Measuring Antigen Suspension. Measure 0.025 ml. of the thoroughly mixed antigen

suspension into a standard tube, the suspension being delivered to the bottom of the tube.

Table 30

PRESUMPTIVE TEST

(Sensitized Antigen)

Ratio of serum: antigen suspension	6 : 1
Antigen suspension, ml.	0.025
Serum, ml.	0.15
Shake by hand for 10 seconds.	
Let stand for three to five minutes.	
Shake for three minutes in mechanical shaker.	
Add saline, ml.	0.5
Shake by hand for 10 seconds.	
Read immediately.	

MEASURING SERUM. Add 0.15 ml. serum which has been heated for 30 minutes at 56° C., and mix with the antigen suspension by shaking the rack vigorously by hand for about 10 seconds. Then the rack is allowed to stand three to five minutes.

SHAKING. The rack is shaken in the usual manner for three minutes in a shaking apparatus.

ADDING SALT SOLUTION. To the tube is added 0.5 ml. physiological salt solution. The contents of the tube are mixed and examined immediately for the presence of flocculation.

INTERPRETATION OF RESULTS. The results are read in the same manner as the standard Kahn test. Complete precipitation, four plus or three plus, is interpreted as positive; a moderate precipitation reaction, two plus, is interpreted as doubtful, while very weak reactions, such as one plus or plus-minus, are classed with the negative reactions. When used as a screen test, every serum that shows any reaction whatsoever with the presumptive Kahn test must be checked by the standard Kahn test.

KOLMER COMPLEMENT-FIXATION TESTS FOR SYPHILIS

Glassware and Apparatus: PIPETS. Accurate pipets are essential. These should include 1-ml. pipets graduated in 0.01 ml. to the tip, 5-ml. pipets graduated in 0.1 ml., and 10-ml. pipets graduated in 0.1 ml. Electrically driven automatic pipets capable of delivering 0.1- to 1.0-ml. volumes with an accuracy within at least 0.02 ml. are convenient for pipetting saline solution, antigen, complement, corpuscle suspension, and hemolysin.

TEST TUBES. These should measure 85 × 13 mm. inside diameter with rounded bottoms and no lips. Galvanized wire racks carrying 12 rows of six tubes each are convenient.

WATER BATH. Any easily regulated water bath is satisfactory; electric baths with automatic thermo-regulators are recommended.

REFRIGERATOR. Any refrigerator capable of maintaining a constant temperature of 6° to 8° C. is satisfactory.

WASHING OF GLASSWARE. All glassware should be chemically clean and preferably sterile. To clean, tubes and flasks should be emptied and rinsed in running tap water; they should be washed inside and outside with soapy water, rinsed several times in running tap water, inverted in wire baskets, and dried in a hot-air oven at about 160° C.

Pipets should be placed after use in a jar or cylinder of clean water with a pad of cotton in the bottom. To clean, pipets are rinsed thoroughly in running tap water. After cleaning, they are placed in a metal box or wire basket and sterilized in the oven.

If glassware becomes cloudy, it is immersed in bichromate cleaning fluid (2 parts potassium bichromate, 3 parts commercial sulfuric acid, and 25 parts water) for 24 hours. It is then rinsed thoroughly in running tap water and washed as described.

Reagents: SALINE SOLUTION. C.P. sodium chloride is dried thoroughly in a hot-air oven. Eight and one-half Gm. are dissolved in 1000 ml. freshly distilled water (0.85 per cent solution). One ml. of a 10 per cent solution of magnesium sulfate in distilled water is added to each 1000 ml. of solution. The resulting solution is filtered into a flask fitted with a gauze-covered stopper.

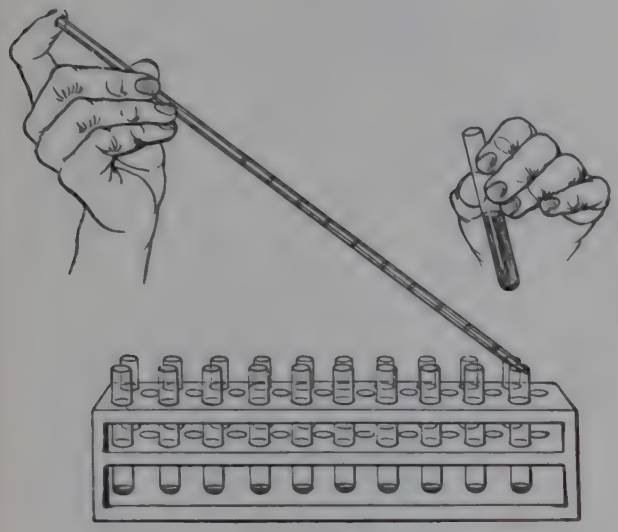
SHEEP CORPUSCLES. Sheep blood is collected by bleeding a sheep from an external jugu-



Diagram to show basic steps in Kahn test procedures. I.

lar vein as aseptically as conditions permit. Collection into an anticoagulant and preservative solution is recommended. For this purpose one part of the following solution (Boerner-Lukens) for each nine parts of blood is satisfactory:

Sodium citrate	8.0 Gm.
Dextrose	20.0 Gm.
1 : 1000 aqueous solution of merthiolate	100.0 ml.



9

PIPETTING SERUM INTO ANTIGEN SUSPENSION



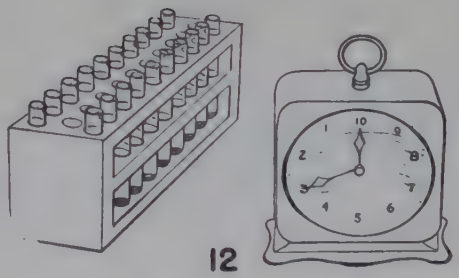
10

BLOWING OUT SERUM



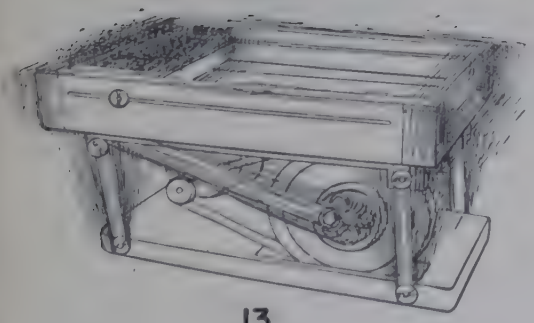
11

SHAKING FOR 10 SEC



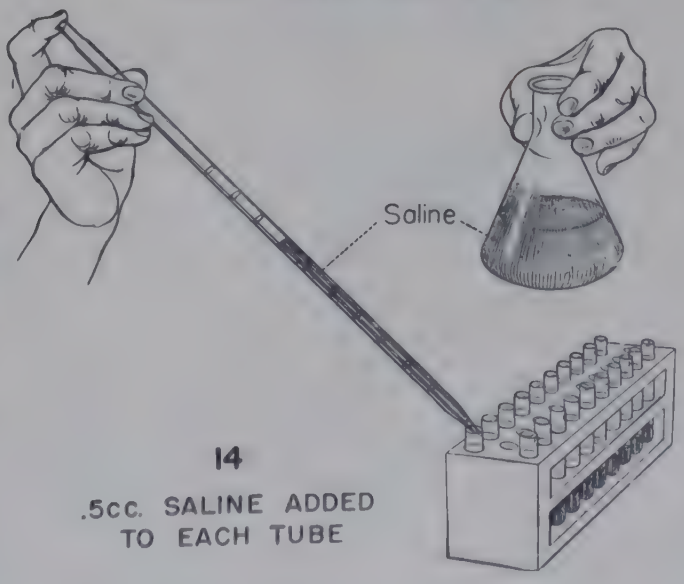
12

TESTS STAND FOR 3 MIN.



13

TESTS SHAKEN FOR 3 MIN



14

.5cc. SALINE ADDED TO EACH TUBE

Diagram to show basic steps in Kahn test procedures. II.

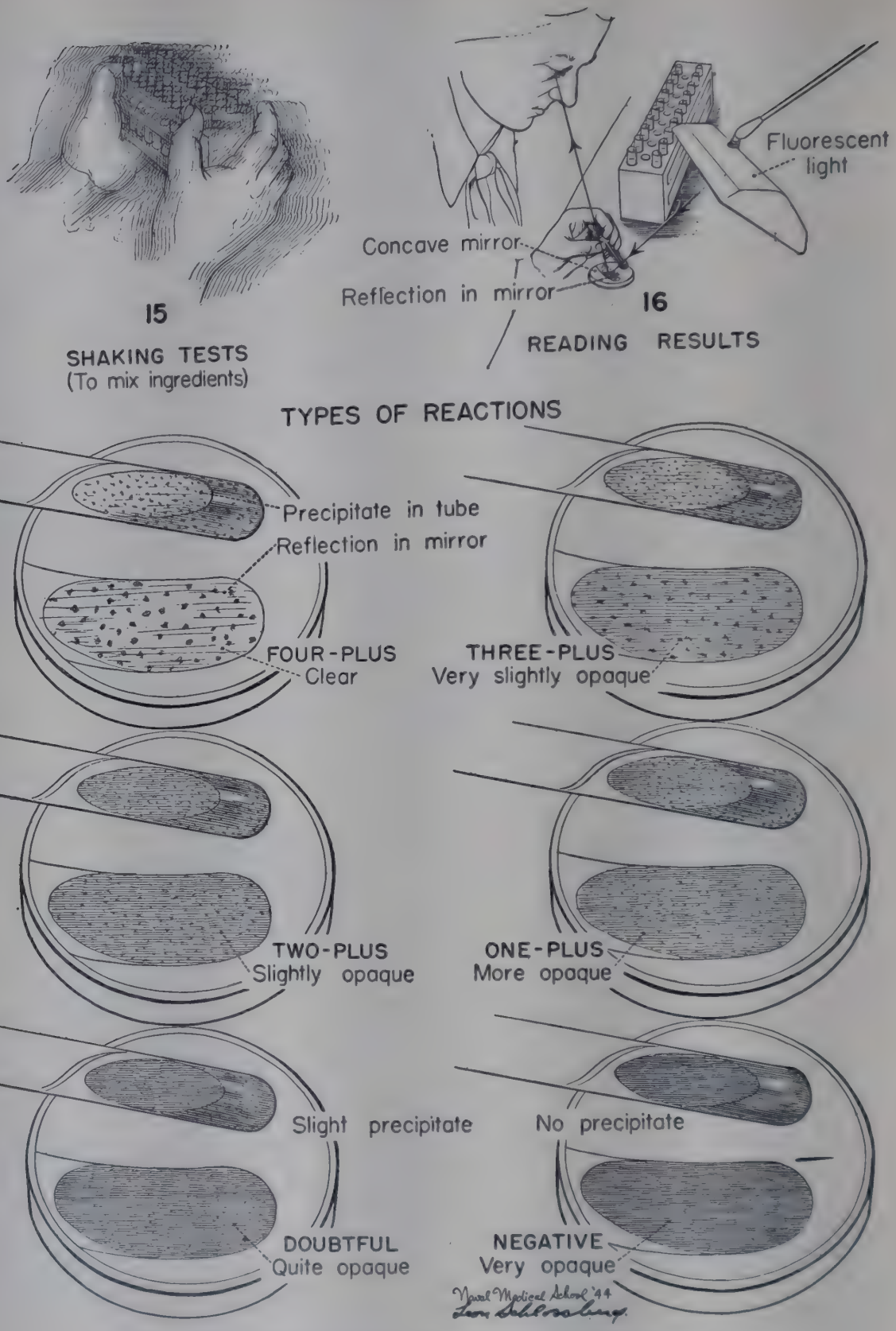


Diagram to show basic steps in Kahn test procedures. III.

Blood collected by this method remains serviceable for one to three weeks if stored at 0° to 5° C. It is advisable to keep the blood in the refrigerator for 48 hours before using.

Sheep's blood may also be preserved conveniently in modified Alsever's solution (Bukantz, Rein, Kent, 1946). The erythrocytes so preserved remain unchanged in their susceptibility to lysis by complement for at least 10 weeks.

Modified Alsever's solution contains:

Dextrose	2.05%
Sodium chloride	0.42%
Trisodium citrate	0.80%
Citric acid	0.055%

A 300-ml. rubber-stoppered bottle is filled to the 150-ml. mark with the solution, evacuated by suction, and sterilized for 15 minutes at 15 pounds pressure. The final sterilized solution has a pH of 6.1 and shows little or no evidence of caramelization. A 20-inch length of rubber tubing fitted with a screw clamp and two 15-stub's-gauge needles is sterilized and used for collecting the sheep's blood into the sterile evacuated bottle. A site over the sheep's jugular vein is sheared and painted with dilute tincture of iodine. After tightening the screw clamp on the rubber tubing, one of the needles is inserted into the rubber stopper of the bottle and the other into the animal's vein. Loosening the screw clamp then permits a rapid flow of blood into the bottle, which should be rotated constantly during the operation. When the bottle is filled to the 300-ml. mark, the screw clamp is tightened and the needles withdrawn from vein and bottle. Blood remaining in the tube should be discarded. Blood and preserving fluid are mixed thoroughly and stored immediately at 3° to 6° C. For convenience in handling, the blood so collected may be dispensed aseptically using a closed system into 60-ml. sterile rubber-stoppered bottles. The rubber stoppers of these bottles are not removed when blood is withdrawn.

To remove blood, the contents of the bottle are mixed thoroughly by gentle rotation. The stopper is swabbed with sterile cotton moistened in alcohol and allowed to dry. Using a sterile syringe and needle, the stopper is perforated and 10 ml. of the blood mixture are transferred to a 15-ml. centrifuge tube. Centrifugation is carried out for 10 minutes at 2000 r.p.m. The supernatant fluid is removed and the packed cells are washed three times with salt solution according to the usual procedure. A 10-ml. sample of the preserved blood should yield approximately 2.5 ml. of packed cells, sufficient for the preparation of 125 ml. of the 2 per cent suspension.

Antisheep Hemolysin. A rabbit is given five to six intravenous injections of 5 ml. of a 10 per cent suspension of washed sheep cells every five days. The rabbit is bled seven to nine days after the last injection if a preliminary titration is satisfactory. The serum (it need not be inactivated) is separated and preserved with an equal part of best-grade neutral glycerin.

Complement. 1. An excellent and economical procedure is to maintain a colony of guinea pigs and to remove 4 to 5 ml. blood from the hearts of a sufficient number to yield the required amount of complement serum. The animals may be bled in rotation every four to six weeks. Instead, three or more pigs may be exsanguinated as follows: The pig is anesthetized lightly with ether or it is stunned with one or two sharp blows on the head; the large blood vessels on both sides of the neck are severed, care being taken not to cut the esophagus or trachea; with the aid of a large funnel the blood is collected in centrifuge tubes. With either method, Kolmer advises placing the blood in an incubator at 37° C. for an hour before the clots are broken up and centrifuged for collection of serum. Otherwise the blood may be kept at room temperature for an hour or two and then placed in the refrigerator overnight when the serum is separated.

2. It is advisable to pretest the serum of each individual guinea pig for nonspecific reactions before using as complement in the conduct of complement-fixation tests for syphilis (Giordano and Carlson, 1939). These preliminary tests should be conducted with the same antigen as employed in the test for syphilis because nonspecific reactions may occur with one antigen and not with another (Harris, 1941). The technic for testing each guinea-pig serum is as follows:

(*a*) A 1 : 30 dilution is prepared by mixing 0.15 ml. with 4.35 ml. saline solution.

(*b*) In a series of six tubes place 0.8, 0.6, 0.4, 0.8, 0.6, and 0.4 ml. respectively of the 1 : 30 dilution of serum.

(*c*) To each of the first three tubes add 0.5 ml. of the same antigen to be used in the complement-titration and the complement-fixation tests so diluted as to carry the optimum dose.

(*d*) Add saline solution to a total volume of 2.0 ml. in each tube, as shown in the following table.

Six tubes for each complement are arranged in the following manner:

	1	*2*	*3*	*4*	*5*	*6*
1 : 30 complement	0.8 ml.	0.6 ml.	0.4 ml.	0.8 ml.	0.6 ml.	0.4 ml.
Kolmer antigen	0.5 ml.	0.5 ml.	0.5 ml.	...	..	..
Saline	0.7 ml.	0.9 ml.	1.1 ml.	1.2 ml.	1.4 ml.	1.6 ml.

(*e*) The contents of the tubes are mixed and the tubes are placed in refrigerator at 6° to 8° C. for 15 to 18 hours, or overnight, at the end of which time they are placed for 10 minutes in a water bath at 37° C.

(*f*) Add 0.5 ml. hemolysin carrying two units and 0.5 ml. of a 2 per cent suspension of sheep cells to each tube.

(*g*) After mixing the contents the tubes are placed in water bath at 37° C. for one hour and examined.

(*h*) Sera showing complete hemolysis in all six tubes are entirely satisfactory. Any serum showing a greater degree of inhibition of hemolysis in the first three tubes carrying the antigen than in the last three tubes not carrying antigen should be regarded as unsatisfactory for the complement-titration and complement-fixation tests insofar as the particular antigen employed is concerned. Such sera may be satisfactory with another Kolmer antigen (Harris, 1941).

3. The most satisfactory method of preserving complement is by evaporation of 5-ml. amounts in separate ampules in the frozen state *in vacuo* by lyophile or the cryochem methods. It usually retains both hemolytic activity and fixability for 8 to 12 months if kept at low temperatures in a refrigerator. On adding 5 ml. distilled water the material goes into immediate solution and is ready for use in the same manner as fresh serum.

Rhamy's sodium acetate method as modified by Sonnenschein (1930) may be employed by adding to complement serum an equal part of solution prepared by dissolving 12 Gm. sodium acetate and 4 Gm. boric acid in 100 ml. distilled water. For use 2 ml. diluted with 8 ml. saline solution gives a 1 : 10 dilution of complement or 1 ml. diluted with 14 ml. saline solution a 1 : 30 dilution. The preserved complement should be kept in a nearly frozen state in the freezing compartment of a mechanical refrigerator. As the complement loses first in fixability by syphilis antigen and reagin, it may not be satisfactory for longer than three or four weeks.

Pooled complement may be divided into unit amounts, as 5 ml., placed in test tubes, and kept in a deep freeze unit. The number of units necessary for one day's tests are then removed daily, and a portion of this complement used in the hemolysin and complement titrations. Complement may be kept in the deep-freeze unit for two to three weeks.

Antigen. A cholesterolized and lecithinized alcoholic extract of powdered beef heart is used. The method of preparation and subsequent titration for standardization may be found in *Approved Laboratory Technic*, by Kolmer and Boerner, fourth edition, D. Appleton-Century Company, 1945. The antigen remains stable for a long period of time if kept at room temperature in a tightly stoppered bottle.

Egg Albumin. When prozone or nonspecific reactions are observed in the tests employing spinal fluid or in quantitative reactions with serum, it may be necessary to add 0.2 ml. of a 50 per cent solution of egg albumin to each tube in the tests. To prepare the egg-albumin solution, a fresh egg is broken and the white is separated from the yolk.

The white is filtered through several layers of gauze, measured, beaten briskly, and an equal volume of saline solution is added to it. The solution is kept in the refrigerator.

An alternate method is to prepare a 10 per cent solution in saline and use this for diluting the complement so that 1 ml. carries two full units.

Sera. 1. All specimens are lined up and properly labeled.

2. All specimens are centrifuged and the sera separated into test tubes properly labeled.

3. The sera are inactivated by placing the tubes in a water bath at 56° C. for 20 to 30 minutes. If the sera have been heated a day or two before the actual test, they should be reheated for five minutes at 56° C. before proceeding with the test.

Spinal Fluid. This is usually tested without any preliminary preparation or inactivation. If over three days old, it may be inactivated 15 minutes to remove thermolabile anticomplementary substances.

Adjustment of Hemolytic System: Titration of Hemolysin. 1. A 2 per cent sheep-corpuscle suspension is prepared. The required amount of preserved sheep's blood is filtered through cotton into a graduated centrifuge tube. Twice as much blood is allowed as the amount of cells required. The blood is centrifuged at a moderate velocity until it is ascertained that all the corpuscles are thrown down. The supernatant fluid is removed with a capillary pipet or by suction. Three to four volumes saline solution are added and mixed by inverting the tube. The mixture is centrifuged until all the corpuscles are again thrown down. This process is repeated for the third time but centrifugation should be continued twice as long as in the first washing in order to pack the cells evenly and firmly. Cells should be washed until the supernatant fluid is almost colorless. Three times are usually sufficient, but if more than four washings are necessary, the cells are too fragile for use.

After the last washing the volume of cells in the graduated centrifuge tube is noted. The supernatant fluid is carefully removed and a 2 per cent suspension is prepared by washing the corpuscles into a flask with 49 volumes saline. The suspension is kept in the refrigerator when not being used. It is always shaken before use to secure an even suspension. The dose is 0.5 ml. of the 2 per cent suspension.

2. For hemolysin and complement titrations a 1 : 30 dilution of complement is prepared by diluting 0.2 ml. complement serum with 5.8 ml. cold saline solution. It is always advisable to dilute complement serum with cold saline solution instead of with saline kept at room temperature. Undiluted and especially diluted complement serum should always be kept in a refrigerator when not in use.

3. A stock dilution of 1 : 100 hemolysin is prepared as follows:

Saline solution	94.0 ml.
Phenol (5 per cent in saline solution)	4.0 ml.
Glycerinized hemolysin (50 per cent)	2.0 ml.

This may be kept in the refrigerator for several months.

4. For the hemolysin titration a 1 : 1000 dilution is prepared by mixing 0.5 ml. of the 1 : 100 stock dilution with 4.5 ml. saline solution. In a series of 10 tubes higher dilutions (with thorough mixing) of hemolysin are prepared as follows:

No. 1.—0.5 ml. hemolysin (1 : 1000) = 1 : 1000
No. 2.—0.5 ml. hemolysin (1 : 1000) + 0.5 ml. saline solution = 1 : 2000
No. 3.—0.5 ml. hemolysin (1 : 1000) + 1.0 ml. saline solution = 1 : 3000
No. 4.—0.5 ml. hemolysin (1 : 1000) + 1.5 ml. saline solution = 1 : 4000
No. 5.—0.5 ml. hemolysin (1 : 1000) + 2.0 ml. saline solution = 1 : 5000
No. 6.—0.5 ml. hemolysin (1 : 3000) + 0.5 ml. saline solution = 1 : 6000
(transferred from tube No. 3)
No. 7.—0.5 ml. hemolysin (1 : 4000) + 0.5 ml. saline solution = 1 : 8000
(transferred from tube No. 4)

No. 8.—0.5 ml. hemolysin (1 : 5000) + 0.5 ml. saline solution = 1 : 10,000
(transferred from tube No. 5)
No. 9.—0.5 ml. hemolysin (1 : 6000) + 0.5 ml. saline solution = 1 : 12,000
(transferred from tube No. 6)
No. 10.—0.5 ml. hemolysin (1 : 8000) + 0.5 ml. saline solution = 1 : 16,000

In a series of 10 tubes the hemolysin titration is set up as shown in the following table:

Table 31

HEMOLYSIN TITRATION

Tube	*Hemolysin 0.5 ml.*	*Complement (1 : 30) (ml.)*	*Saline Solution (ml.)*	*Corpuscles (ml.)*
1	1 : 1000	0.3	1.7	0.5
2	1 : 2000	0.3	1.7	0.5
3	1 : 3000	0.3	1.7	0.5
4	1 : 4000	0.3	1.7	0.5
5	1 : 5000	0.3	1.7	0.5
6	1 : 6000	0.3	1.7	0.5
7	1 : 8000	0.3	1.7	0.5
8	1 : 10,000	0.3	1.7	0.5
9	1 : 12,000	0.3	1.7	0.5
10	1 : 16,000	0.3	1.7	0.5

The contents of each tube are mixed and incubated in the water bath at 38° C. for one hour. The unit of hemolysin is read. *The unit is the highest dilution of hemolysin that gives complete hemolysis.*

Two units are used in the complement and antigen titrations and in the complement-fixation tests. Hemolysin is so diluted that 0.5 ml. contains two units. For example, if the unit equals 0.5 ml. of 1 : 6000, two units equal 0.5 ml. of 1 : 3000. Just enough hemolysin for the complement-titration and the complement-fixation tests is diluted.

The following table shows how the dilutions are made so that 0.5 ml. carries two units.

Table 32

DILUTION OF HEMOLYSIN

1 Unit 0.5 ml. of—	*2 Units Would Be 0.5 ml. of—*	*Prepared by Diluting 1 ml. of Stock 1 : 100 with Following Amounts of Saline (ml.)*
1 : 1000	1 : 500	4
1 : 2000	1 : 1000	9
1 : 3000	1 : 1500	14
1 : 4000	1 : 2000	19
1 : 5000	1 : 2500	24
1 : 6000	1 : 3000	29
1 : 8000	1 : 4000	39
1 : 10,000	1 : 5000	49

Hemolysin of high titer is recommended, and the unit should be 0.5 ml. of 1 : 4000 or higher.

In practice the hemolysin titration may be placed in the water bath at the same time as the complement titration; at the end of the first incubation of the complement titration the unit of hemolysin is available and two units are added to all tubes of the complement titration, etc.

TITRATION OF COMPLEMENT. Antigen dilution is prepared for the complement-titration and the complement-fixation tests. The antigen is diluted according to titer (previously determined and stated on antigen bottle) so that the dose employed is contained in 0.5 ml. This dilution is prepared by placing the required amount of saline solution in a flask and adding the required antigen drop by drop, shaking the flask after each addition of antigen. For example, if titer is 1 : 600, 0.1 ml. antigen is added to 60 ml. saline. Both the undiluted antigen and antigen dilution are always kept at room temperature.

In a series of eight test tubes set up, the complement titration is as follows:

Table 33

COMPLEMENT TITRATION

Tube	*Complement (ml.) (1 : 30)*	*Antigen Dose (ml.)*	*Saline Solution (ml.)*	*Water Bath 37° C.*	*Hemolysin (ml.) (2 Units)*	*Corpuscles (ml.) (2%)*	*Water Bath 37° C.*
1	0.2	0.5	1.3		0.5	0.5	
2	0.25	0.5	1.3	for	0.5	0.5	for
3	0.3	0.5	1.2		0.5	0.5	
4	0.35	0.5	1.2	one	0.5	0.5	one
5	0.4	0.5	1.1		0.5	0.5	
6	0.45	0.5	1.1	hour	0.5	0.5	hour
7	0.5	0.5	1.0		0.5	0.5	
8	None	None	2.5		None	0.5	

The smallest amount of complement just giving complete sparkling hemolysis is the "exact unit." The next higher tube is the "full unit" which contains 0.05 ml. more complement. In conducting the antigen-titration and complement-fixation tests, two full units are employed and so diluted as to be contained in 1 ml. as per the following example:

Exact unit ..... 0.30 ml.
Full unit ..... 0.35 ml.
Dose (two full units) ..... 0.70 ml.

To calculate the dilution to employ so that 1 ml. contains the dose of two full units, 30 is divided by the dose: $\frac{30}{0.7} = 43$ or 1 ml. of 1 : 43 dilution of serum.

The following table gives additional examples:

Table 34

DILUTION OF COMPLEMENT

Exact Unit (ml.)	*Full Unit (ml.)*	*Two Full Units (ml.)*	*Dilution to Use*	*Preparation*
0.20	0.25	0.5	1 : 60	1 ml. serum 59 ml. saline
0.25	0.30	0.6	1 : 50	1 ml. serum 49 ml. saline
0.30	0.35	0.7	1 : 43	1 ml. serum 42 ml. saline
0.35	0.40	0.8	1 : 37	1 ml. serum 36 ml. saline
0.40	0.45	0.9	1 : 33	1 ml. serum 32 ml. saline
0.45	0.50	1.0	1 : 30	1 ml. serum 29 ml. saline
0.50	0.55	1.1	1 : 27	1 ml. serum 26 ml. saline

If all tubes show complete hemolysis, 0.3 ml. is taken as the exact unit, as less complement falls below the absolute minimum and is likely to give incomplete hemolysis and prove unsatisfactory.

Choice of Methods. The regular quantitative method employs five doses of serum or spinal fluid and is preferred when conditions permit, especially in testing the sera and spinal fluids of patients under treatment for syphilis.

A three-tube qualitative test may be conducted with two doses of serum (0.2 and 0.1 ml.). In the interest of economy, both tests may be conducted by using reagents in one-half amounts. This economy applies not only to the amounts of serum or spinal fluid but to the amounts of complement, antigen, hemolysin, and sheep cells employed.

Quantitative Complement-fixation Test. 1. For each serum six test tubes are arranged and in them are placed the following amounts of saline solution, respectively: 0.9, 0.5, 0.5, 0.5, 2.0, and 0.5 ml. To the first tube 0.6 ml. inactivated serum is added. The contents of this tube are mixed and 0.5 ml. is transferred to No. 2 and 0.5 ml. to No. 6 (control tube). The contents of No. 2 are mixed and 0.5 ml. is transferred to No. 3. No. 3 is mixed and 0.5 ml. transferred to No. 4. No. 4 is mixed, 0.5 ml. transferred to No. 5, and 2.0 ml. discarded after mixing.

2. For each specimen of spinal fluid six test tubes are arranged and 0.5 ml. saline solution is placed in tubes 2, 3, 4, 5, and 6. To Nos. 1, 2, and 6 is added 0.5 ml. spinal fluid. The contents of tube No. 2 are mixed and 0.5 ml. transferred to No. 3. No. 3 is mixed and 0.5 ml. transferred to No. 4. No. 4 is mixed and 0.5 ml. transferred to No. 5. No. 5 is mixed and 0.5 ml. discarded.

To each of six tubes and to the antigen control is added 0.2 ml. egg albumin (50 per cent). (This may usually be omitted if pretested complement is used.)

3. To the first five tubes of each test series (serum or spinal fluid) 0.5 ml. diluted antigen carrying the proper dose is added and mixed thoroughly.

4. The tubes are allowed to stand for 10 to 30 minutes at room temperature. If a longer interval elapses the racks are placed in the refrigerator.

5. To all tubes 1 ml. complement (two full units) is added.

6. The following controls are included: (*a*) *Antigen control* containing 0.5 ml. diluted antigen, 0.5 ml. saline solution, 1 ml. complement (two full units), and 0.2 ml. egg albumin (50 per cent) if it has been used in the tests.

(*b*) *Hemolytic system control* containing 1 ml. saline solution and 1 ml. diluted complement (two full units).

(*c*) *Corpuscle control* containing 2.5 ml. saline solution.

(*d*) Controls of *positive* and *negative* sera are advisable.

7. The contents of each tube are mixed by gentle shaking and the tubes placed in the refrigerator at 6° or 8° C. for 15 to 18 hours.

8. The tubes are placed in water bath at 37° C. for 10 minutes (not longer).

9. To all tubes, except the corpuscle control, are added 0.5 ml. hemolysin (carrying two units) and 0.5 ml. of 2 per cent cell suspension which has been well mixed.

10. The contents of each tube are mixed by gentle but thorough shaking and the tubes placed in water bath at 37° C. *The serum, antigen, and hemolytic system controls are watched and 10 minutes after these show complete hemolysis (usually 25 to 30 minutes) the tubes are removed and readings made.*

11. Table 35 shows the set-up for the quantitative complement-fixation test with serum.

12. Table 36 shows the set-up for the quantitative complement-fixation test with spinal fluid.

Three-tube Qualitative Test. Three test tubes are arranged and 0.9, 0.5, and 0.5 ml. saline solution are placed in each respectively. To tube 1 is added 0.6 ml. serum. After mixing 0.5 ml. is transferred to tube 2 and a like amount to tube 3 (control). The contents of tube 2 are mixed and 0.5 ml. is discarded. Each tube now carries 0.5 ml. containing 0.2, 0.1, and 0.2 ml. serum respectively. Antigen (0.5 ml.) is added to tubes 1 and 2 and the test completed in the same manner as the six-tube quantitative test. The same controls are used as in the quantitative test.

Table 35

QUANTITATIVE COMPLEMENT-FIXATION TEST WITH SERUM

Tube	*Serum (in 0.5 ml.)*	*Antigen (ml.)*	Interval of 10 to 30 minutes at room temperature.	*Complement (ml.) (2 Full Units)*	Primary incubation in refrigerator at 6° to 8° C. for 15 to 18 hr. followed by 10 to 15 min. 37° C.	*Hemolysin (ml.) (2 Units)*	*Corpuscles (ml.) (2%)*	Secondary incubation in water bath at 37° C.
1	0.2 ml.	0.5		1.0		0.5	0.5	
2	0.1 ml.	0.5		1.0		0.5	0.5	
3	0.05 ml.	0.5		1.0		0.5	0.5	
4	0.025 ml.	0.5		1.0		0.5	0.5	
5	0.005 ml.	0.5		1.0		0.5	0.5	
6	0.2 ml. (control)	None		1.0		0.5	0.5	
Controls	Antigen control: 0.5 ml. saline	0.5		1.0		0.5	0.5	
	Hemolytic control: 1.0 ml. saline	None		1.0		0.5	0.5	
	Corpuscle control: 2.5 ml. saline	None		None		None	0.5	

Table 36

QUANTITATIVE COMPLEMENT-FIXATION TEST WITH SPINAL FLUID

Tube	*Spinal Fluid (in 0.5 ml. volume)*	*Egg Albumin* 50% (ml.)*	*Antigen (ml.)*	Interval of 10 to 30 min. at room temperature.	*Complement (ml.) (2 Full Units)*	Mix all tubes. Primary incubation 6° to 8° C. for 15 to 18 hr. Then 10 to 15 min. in 37° C. water bath.	*Hemolysin (ml.)*	*Corpuscles 2% (ml.)*	Mix all tubes. Secondary incubation in water bath at 37° C.
1	0.5 ml.	0.2	0.5		1.0		0.5	0.5	
2	0.25 ml.	0.2	0.5		1.0		0.5	0.5	
3	0.125 ml.	0.2	0.5		1.0		0.5	0.5	
4	0.0625 ml.	0.2	0.5		1.0		0.5	0.5	
5	0.03125 ml.	0.2	0.5		1.0		0.5	0.5	
6	0.5 ml. (control)	0.2	None		1.0		0.5	0.5	
Controls	Antigen control: 0.5 ml. saline	0.2	0.5		1.0		0.5	0.5	
	Hemolytic control: 1.0 ml. saline	None	None		1.0		0.5	0.5	
	Corpuscle control: 2.5 ml. saline	None	None		None		None	0.5	

*Can usually be omitted if pretested complement is employed.

ONE-HALF-QUANTITY METHODS. The hemolysin and complement are titrated and diluted in the same manner as in the regular methods. The dose of the former (carrying two units) is 0.25 instead of 0.5 ml. and the dose of the latter (carrying two full units) is 0.5 ml. instead of 1.0 ml. The dose of 2 per cent corpuscle suspension is 0.25 instead of 0.5 ml.

1. For each quantitative serum test: (*a*) Six test tubes are arranged and the following amounts of saline solution are placed in each respectively: 0.6, 0.3, 0.3, 0.3, 1.2, and 0.3 ml.

(*b*) To tube 1, 0.3 ml. serum is added. After mixing, 0.3 ml. is transferred to tube 2 and to tube 6. The contents of tube 2 are mixed and 0.3 ml. is transferred to tube 3, and so on to tube 5 from which 1.2 ml. are discarded after mixing.

(*c*) The test is completed including antigen, hemolytic system, and corpuscle controls as shown in the following table. It is advisable to include tests with known positive and negative sera as controls.

Table 37

QUANTITATIVE COMPLEMENT-FIXATION TEST WITH SERUM—ONE-HALF-QUANTITY METHOD

Tube	*Serum (in 0.3 ml.)*	*Antigen (ml.)*	Interval of 10 to 30 minutes at room temperature.	*Complement (ml.) (2 Full Units)*	Mix all tubes. Primary incubation 6° to 8° C. for 15 to 18 hr. Then 10 to 15 min. in water bath at 37° C.	*Hemolysin (ml.) (2 Units)*	*Corpuscles (ml.) (2%)*	Mix all tubes. Secondary incubation in water bath at 37° C.
1	0.1 ml.	0.25		0.5		0.25	0.25	
2	0.05 ml.	0.25		0.5		0.25	0.25	
3	0.025 ml.	0.25		0.5		0.25	0.25	
4	0.0125 ml.	0.25		0.5		0.25	0.25	
5	0.0025 ml.	0.25		0.5		0.25	0.25	
6	0.1 ml. (control)	None		0.5		0.25	0.25	
Control 1	Antigen control: 0.3 ml. saline	0.25		0.5		0.25	0.25	
Control 2	Hemolytic control: 0.5 ml. saline	None		0.5		0.25	0.25	
Control 3	Corpuscle control: 1.3 ml. saline	None		None		None	0.25	

2. For the three-tube qualitative test: (*a*) Three tubes are arranged and 0.6 ml. of saline is placed in tube 1, and 0.3 ml. in tubes 2 and 3.

(*b*) To tube 1 is added 0.3 ml. of inactivated serum. After mixing, 0.3 ml. is transferred to tube 2 and to tube 3. The contents of tube 2 are mixed and 0.3 ml. is discarded. Antigen (0.25 ml.) is added to tubes 1 and 2 and the test completed in the same manner as in the six-tube quantitative test. The same controls are used.

3. For each quantitative spinal fluid test: (*a*) Six test tubes are arranged and 0.25 ml. saline solution is placed in each of tubes 2, 3, 4, 5, and 6.

(*b*) To tubes 1, 2, and 6 is added 0.25 ml. spinal fluid.

(*c*) The contents of tube 2 are mixed and 0.25 ml. is transferred to tube 3, and so on to tube 5 from which 0.25 ml. is discarded after mixing.

(*d*) To all tubes including the antigen control, 0.1 ml. of 50 per cent solution of egg albumin is added. (This may usually be omitted if pretested complement is used.)

(*e*) The test is completed with antigen, hemolytic system, and corpuscle controls as shown in the following table. It is advisable to include tests with known positive and negative spinal fluids as controls.

4. The secondary incubation is conducted and the reactions read as described above for the regular methods.

Table 38

QUANTITATIVE COMPLEMENT-FIXATION TEST WITH SPINAL FLUID—ONE-HALF-QUANTITY METHOD

Tube	*Spinal Fluid (in 0.25 ml. volume)*	*Egg Albumin* (50%) (ml.)*	*Antigen (ml.)*	Interval of 10 to 30 min. at room temperature.	*Complement (2 Full Units) (ml.)*	Mix all tubes. Primary incubation 6° to 8° C. for 15 to 18 hr. Then 10 to 15 min. in water bath at 37° C.	*Hemolysin (2 Units) (ml.)*	*Corpuscles (2%) (ml.)*	Mix all tubes. Secondary incubation in water bath at 37° C.
1	0.25 ml.	0.1	0.25		0.5		0.25	0.25	
2	0.125 ml.	0.1	0.25		0.5		0.25	0.25	
3	0.0625 ml.	0.1	0.25		0.5		0.25	0.25	
4	0.03125 ml.	0.1	0.25		0.5		0.25	0.25	
5	0.015625 ml.	0.1	0.25		0.5		0.25	0.25	
6	0.25 ml. (control)	0.1	None		0.5		0.25	0.25	
Control 1	Antigen control: 0.5 ml. saline	0.1	0.25		0.5		0.25	0.25	
Control 1	Hemolytic control: 0.5 ml. saline	None	None		0.5		0.25	0.25	
Control 1	Corpuscle control: 1.25 ml. saline	None	None		None		None	0.25	

*Can usually be omitted if pretested complement is employed.

Reactions and Interpretation. All serum, spinal fluid, antigen, and hemolytic controls should show complete hemolysis. The corpuscle controls should show no hemolysis. The reactions should be recorded for each tube of complement fixation tests as: ++++ (4), +++ (3), ++ (2), + (1), —.

REACTIONS—QUANTITATIVE TESTS. The reactions in the quantitative or six-tube tests with serum or spinal fluid may be interpreted as follows:

1. *Very strongly positive* when complete fixation occurs in any of the first three, four, or five tubes, like 344 — —, 444 — —, 4442 —, 4443 —, 44444.

2. *Strongly positive* when complete fixation (++++) occurs in the second tube, like 4431 —, 442 — —, 342 — —, or 44 — — —.

3. *Moderately positive* when complete fixation (++++) occurs in the first tube only, like 431 — —, 42 — — —, or 4 — — — —.

4. *Weakly positive* when partial fixation occurs in one or more tubes, like 321 — —, 21 — — —, or 1 — — — —.

5. *Doubtful* when the reaction is ± in the first tube, like ± — — — —.

6. *Negative* when there is complete hemolysis in all tubes, — — — — —.

Otherwise the results may be reported in Kolmer units in which the potency of any serum or spinal fluid is determined according to the formula S=4D as used in the Kahn quantitative serum test, where S is the serum or spinal fluid potency in terms of units and D is the highest dilution giving a positive four-plus (++++), three-plus (+++), two-plus (++), or one-plus (+) reaction.

If a serum gives a ++++, +++, ++, or + reaction in the first tube only and the

reaction is negative in the remaining four tubes, the serum is considered as containing Kolmer units as indicated by the plus signs (4 units, 3 units, 2 units, or 1 unit, respectively). If a reaction of any degree occurs in the second tube (D = 2) and no reaction in the remaining tubes, the serum is considered as containing eight Kolmer units; if a reaction of any degree occurs in the third tube (D = 4) and none in the last tube, it is considered as containing 16 Kolmer units; if a reaction of any degree occurs in the fourth tube (D = 8) and there is no reaction in the last tube, it is considered as containing 32 Kolmer units; if a reaction of any degree occurs in the fifth tube (D = 40), it is considered as containing 160 or more Kolmer units. In this latter case still higher dilutions of serum may be tested if desired.

If a spinal fluid gives a ++++, +++, ++, or + reaction in the first tube only and none in the remaining tubes, it is considered as containing Kolmer units as indicated by the plus signs (4 units, 3 units, 2 units, or 1 unit, respectively). If any degree of reaction occurs in the second tube (D = 2) and none in the remaining tubes, it is considered as containing eight Kolmer units; if a reaction of any degree occurs in the third tube (D = 4) and not in the remaining tubes, it is considered to contain 16 Kolmer units; if a reaction of any degree occurs in the fourth tube and not in the fifth, it is considered to contain 32 units; if a reaction occurs in the fifth tube (D = 16), it is considered as containing 64 or more Kolmer units. In this latter case still higher dilutions of spinal fluid may be tested if desired.

Weakly anticomplementary reaction may be safely reported as follows: 4441 — 2 = positive; 441 — — 1 = positive; 441 — — ± = positive; 32 — — — ± = positive; 441 — — 3 = doubtful; 32 — — — 1 = doubtful; 21 — — — ± = doubtful; 3 — — — — ± = doubtful; 1 — — — — ± = negative; 1 — — — — 1 = negative; 2 — — — — 1 = negative; 2 — — — — 2 = negative.

Reactions—Qualitative Test. The reaction in the three-tube qualitative test may be interpreted as follows:

1. *Strongly positive* when complete fixation (++++) occurs in the second tube, like 44 or 34.
2. *Moderately positive* when complete fixation (++++) occurs in the first tube only, like 42 or 41.
3. *Weakly positive* when partial fixation occurs in one or both tubes, like 31, 21, 3—, 2—, or 1—.
4. *Doubtful* when the reaction is ± in the first tube, like ± —.
5. *Negative* when there is complete hemolysis in both tubes, — —.

Analysis of Difficulties: Difficulties Due to Complement. When difficulties are experienced they are usually first ascribed to defective hemolysin or antigen, but since both of these keep very well they are rarely responsible. In some cases difficulties are due to the use of complement too low in hemolytic activity and particularly in the case of preserved complement. This is especially likely to be the case during the hot months of the year and likewise when the sera of underweight or pregnant guinea pigs are used, as well as of animals previously employed in inoculation tests. Under these conditions anticomplementary reactions may occur with incomplete hemolysis of the antigen, serum, and spinal-fluid controls. When the unit of complement is more than 0.5 ml. of 1 : 30 dilution in the regular tests it should not be used.

Furthermore, although the complement may be satisfactory from the standpoint of hemolytic activity, it may be defective because it is supersensitive to what may be called the occult anticomplementary effects of antigen, serum, or spinal fluid. Under these circumstances inhibition of hemolysis occurs in those tubes carrying antigen while the antigen, serum, and spinal-fluid controls show complete hemolysis, causing danger of reporting falsely positive or nonspecific reactions. When pretested complement or the pooled complement of a large number of guinea pigs is employed false or nonspecific

reactions are exceptional occurrences. However, since they may happen in quantitative and qualitative spinal-fluid tests, it is recommended that egg albumin be used routinely in the conduct of these tests, especially if pretested complement is not being used.

Difficulties Due to Prozone Reactions. Prozone reactions may occur in quantitative tests with sera, giving reactions like — — 234, with complete hemolysis of the serum control, but sometimes with incomplete hemolysis of the antigen control. They occur quite infrequently when pretested or pooled complement is being used. They may be prevented by the use of 50 per cent egg albumin.

Difficulties Due to Hemolysin. As previously stated, defective hemolysin is usually first suspected but is usually least likely to be the cause of difficulty, especially if the hemolysin has been previously found satisfactory. The unit of anti-sheep hemolysin in the regular tests should be not greater than 0.5 ml. of 1 : 4000 and hemolysins of this and higher strength are so easy to prepare that it is a mistake to use weaker products. If the saline solution and complement are satisfactory, a good hemolysin is rarely responsible even when shipped over long distances or kept in a refrigerator over months or even years of time.

Difficulties Due to Corpuscles. When blood is obtained from an abattoir, one is almost certain sooner or later to encounter the corpuscles of occasional animals possessing increased resistance to serum hemolysis. The cause of this phenomenon is unknown, but fortunately it is rare. The remedy is to discard the corpuscles and secure a fresh supply of blood. When the corpuscles of preserved blood tend to become too fragile, it is advisable to use 0.9 per cent saline instead of the usual 0.85 per cent in the conduct of the tests.

Difficulties Due to Antigen. Provided no mistakes have occurred in dilution and dosage, antigen is very rarely the cause of trouble. When the antigen control shows incomplete hemolysis, it is almost surely due to some component of the hemolytic system, especially complement supersensitive to antigen, in which case egg albumin may be used.

Difficulties Due to Anticomplementary Sera and Spinal Fluids. Sera and spinal fluid may be found to be anticomplementary, as shown by incomplete hemolysis of the controls. After experience has been gained, some of these reactions may be safely read, as stated, but as a general rule it is much safer and wiser to repeat the tests with fresh serum or spinal fluid, especially if the technician lacks experience in complement-fixation work. It is infinitely better to repeat the test than to run the slightest chance of error, especially the regrettable error of reporting a false positive reaction. Sometimes the majority of sera or spinal fluids of a day's work show incomplete hemolysis of the controls, but this trouble is not due to anticomplementary effects on the part of the sera or fluids but rather to the use of a defective supersensitive complement. Under these conditions the tests may have to be repeated, and for this reason the unused portion of the sera and of spinal fluids should always be kept in the refrigerator until the test is completed in case repetitions are required. In case difficulties are due to the thermostable anticomplementary substances in sera, the sera can usually be satisfactorily tested after preparation by Sach's method including use of egg albumin.

Cardiolipin Antigens in Serodiagnostic Tests for Syphilis

The production of standard lipoidal antigens for the various serologic diagnostic tests for syphilis has been a difficult problem. Even though the directions as described by the respective authors are exactly followed under carefully controlled conditions, it is not always possible to produce antigens of constant reactivity. The isolation by Pangborn of cardiolipin which she identifies as the substance "essential for the reactivity of beef heart antigens in the serologic tests for syphilis" has

made it possible to prepare reproducible antigens which may be more easily adjusted to a desired level of sensitivity. A great deal of work has yet to be done before a complete evaluation of tests employing cardiolipin antigens can be formulated. The present knowledge is reviewed in the following paragraphs by Pangborn (personal communication, 1946).

The isolation from beef heart of the phospholipid to which the name "cardiolipin" has been given was first reported in 1941, and the details of improved methods for its preparation were subsequently described (Pangborn, 1941–1947). This substance is an essential component of the tissue-extract antigens which have been widely used in the serodiagnosis of syphilis. Antigens prepared with pure cardiolipin compare very favorably with the crude and difficultly reproducible extract antigens.

Cardiolipin is apparently not serologically active alone, but requires the admixture of purified lecithin, which by itself manifests no serologic activity. No explanation of this combined effect of the two phospholipids is as yet available. The addition of cholesterol increases the reactivity of the mixture. These general considerations apply both to complement-fixation and flocculation procedures. The proper adjustment of cardiolipin antigens for a particular serologic technic requires determination of optimal proportion of cardiolipin, lecithin, and cholesterol in the mixture (Harris and Portnoy, 1944; Maltaner and Maltaner, 1945, 1946; Rein and Bossak, 1946; Kline, 1946, 1947; Harris, 1946; Brown, 1946, 1947; Kahn, 1946). Standardization of successive lots of antigen is then unnecessary, since the ingredients are essentially pure and different preparations do not vary significantly in chemical and serologic properties. The availability of an essentially pure antigen should facilitate the more uniform standardization of the various serologic tests for syphilis.

Preliminary data on the constitution of cardiolipin have been reported (Pangborn, 1946); the substance is best described as a complex phosphatidic acid. It contains no nitrogen. In tissue extracts it occurs in the form of neutral salts, probably of sodium or potassium. Processes for its isolation and purification are based on the formation of less soluble salts, such as those of barium or cadmium, which can readily be precipitated from tissue extracts leaving the bulk of the impurities in solution. These salts may then be purified by repeated precipitation from various organic solvents and the purified material finally reconverted to the alcohol-soluble sodium salt, which is conveniently used in preparing antigen mixtures. The degree of purity attainable is surprisingly high for this type of compound, as judged by constancy of properties on repeated purification and the ease with which identical products can be obtained in successive preparations. Although cardiolipin is highly unsaturated, having an iodine number of about 125, it is stable in alcoholic solution and samples have been kept for periods up to two and a half years with no detectable alteration.

TESTS EMPLOYING CARDIOLIPIN ANTIGEN

The reactivity of tests employing antigens composed of different combinations of cardiolipin, lecithin, and cholesterol is being extensively studied in a number of different serologic procedures. A slide test employing cardiolipin antigen, and a formula for a cardiolipin antigen which may be used in the Kolmer complement-fixation test for syphilis, both formulated at the Venereal Disease Research Laboratory, U. S. Marine Hospital, Staten Island, N. Y., are presented in the following pages. Final evaluation of these procedures has not been completed and must await statistical analysis of results to be accumulated during a longer period.

V.D.R.L. Slide Test

Glassware and Apparatus. The following glassware and apparatus are needed for the performance of the cardiolipin slide test:

1. Pipets: 5-ml., graduated in 0.1 ml. to tip.
   1-ml., graduated in 0.01 ml. to tip.
2. Glass slides (2 × 3 inches) with 12 paraffin rings of approximately 15-mm. inside diameter as described by Kline (1932).
3. Hand or automatic electric-type ringmakers.
4. Slide-holders. Any convenient type holding from one to four slides.
5. Round bottles of 30-ml. capacity with glass or screw-cap stoppers.
6. One- or 2-ml. syringe with 23-gauge, long-bevel hypodermic needle.
7. Serum-tube racks.
8. Serum tubes. Any convenient size to fit the tube racks.
9. Water bath (56° C.).
10. Standard-type centrifuge.
11. Microscope.
12. Boerner-type rotator (optional).

Method of Cleaning Glassware. All glassware should be chemically clean. Serum tubes and pipets are rinsed with tap water, washed thoroughly with a detergent such as Orvus, thoroughly rinsed, allowed to stand overnight in cleaning solution, and then rinsed five times in tap water and three times in distilled water, and dried in a drying oven.

New glass slides (2 × 3 inches) are cleaned with Bon Ami which is removed with a soft cloth after drying. Previously used slides are first freed of paraffin, washed with soap, rinsed free of soap, and then treated like the new slides.

Reagents: Antigen. Antigen for this test contains 0.03 per cent cardiolipin, approximately 0.27 per cent purified lecithin, and 0.9 per cent cholesterol in absolute alcohol. These percentages of cardiolipin and lecithin are gravimetric equivalents calculated from chemical assays, but each lot of cardiolipin must be serologically standardized by proper comparison with an antigen of known reactivity.

Antigen is dispensed in screw-cap (tin-foil or vinylite lined) bottles and stored in the "dark." The components of this antigen remain in solution at usual room temperatures, so any precipitate noted will indicate changes due to other factors such as evaporation or foreign materials contributed by pipets. Antigen containing precipitate should be discarded.

Serum. Clear serum, obtained by centrifuging whole clotted blood, is heated at 56° C. for 30 minutes before being tested. All sera are examined when removed from the water bath and those found to contain particulate debris are recentrifuged. Serums to be tested more than four hours after being heated should be reheated at 56° C. for 10 minutes.

Saline. Buffered saline containing 1 per cent sodium chloride is prepared as follows:

Formaldehyde, neutral, reagent grade	0.50 ml.
Secondary sodium phosphate (Na_2HPO_4, $12H_2O$)	0.093 Gm.
Primary potassium phosphate (KH_2PO_4)	0.17 Gm.
Sodium chloride, A.C.S.	10.00 Gm.
Distilled water	1000.00 ml.

Preparation and Preliminary Testing of Antigen Emulsion: Preparation of Antigen Emulsion. To the bottom of a 30-ml., round, glass- or screw-cap-stoppered bottle pipet 0.4 ml. of buffered saline solution. From the lower portion of a 1-ml. pipet (graduated to the tip) add 0.5 ml. antigen, drop by drop (rapidly without long interval between drops), directly onto the saline while continuously rotating the bottle on a flat surface. The last drop of antigen is blown from the pipet without touching it to the saline and

rotation is continued for 10 more seconds. Then 4.1 ml. buffered saline are added from a 5-ml. pipet, the top is placed on the bottle, and the bottle shaken vigorously (throwing liquid from top to bottom of bottle) for approximately 10 seconds. Antigen emulsion is then ready for use and may be used during one day. The amount (5.0 ml.) is sufficient for approximately 250 serum tests.

Testing Antigen Emulsion Delivery Needle. The number of antigen particles per microscopic field is determined by the size of the drop of antigen emulsion used. For this reason the needle used each day should be checked.

Antigen emulsion is dispensed from a 23-gauge, long-bevel, hypodermic needle attached to a 1- or 2-ml. syringe which is allowed to stand in the antigen-emulsion bottle when not in use. Approximately 60 drops should be obtained from 1 ml. of antigen emulsion. This can be accomplished by holding the syringe so that the needle bevel is down and the dropping surface horizontal. Increasing the angle at which the syringe is held diminishes the dropping surface and consequently decreases the size of the drop. When allowed to stand, antigen emulsion should be gently mixed before use by rotating the bottle and filling and emptying the syringe.

Preliminary Testing of Antigen Emulsion. Each preparation of an antigen emulsion should first be examined by testing known positive and negative sera. This is accomplished by adding one drop of antigen emulsion to 0.05 ml. of each serum and completing the test as described under "Qualitative Serum Test." These tests should present typically positive and negative results, respectively, and the number of antigen particles per microscopic field should be optimum.

Qualitative Serum Test: Test Procedure. 1. Into one ring of a paraffin-ringed glass slide pipet 0.05 ml. heated serum.

2. Add one drop (approximately 1/60 ml.) antigen emulsion to each serum.

3. Rotate the slides for four minutes. (If rotated by hand on a flat surface, this movement should roughly circumscribe a 2-inch circle 120 times a minute. The Boerner-type rotator set at 180 r.p.m. may also be used for this test.)

4. Reactions are read immediately after rotation.

Reading and Reporting Test Results. Tests are read microscopically, with low-power objective, at 100 × magnification. The antigen particles appear as short-rod forms at this magnitude. Aggregation of these particles into large or small clumps is interpreted as indicating the degree of positivity.

Reading	Report
No clumping or very slight roughness	Negative (N)
Small clumps	Weakly positive (W.P.)
Medium and large clumps	Positive (P)

Proper reading and interpretation of the results require that the technician have had training and experience in each technic employed, but rough equivalents may be used for describing similarities between the reading of this and other slide tests. The *weakly positive* range of reaction in this test includes clumpings similar to those reported as plus-minus or one plus and the *positive* range those reported as two plus, three plus, or four plus in other slide tests. However, it is recommended that these numerals not be used for reporting results of this test.

Zonal reactions, due to an excess of reactive serum component, are recognized by irregular clumping and the loosely bound appearance of the clumps. The usual positive reaction is characterized by large and small clumps of fairly uniform size, and experience will allow differentiation to be made between this type of reaction and the zonal picture wherein large and/or small clumps may be intermingled with free antigen particles.

Whenever a zonal-type reaction is suspected the serum in question should be diluted 1 : 5 and 1 : 25 and retested. Dilutions may be prepared by placing 0.4 ml. saline in

each of two test tubes, adding 0.1 ml. heated serum to tube 1, mixing well, and transferring 0.1 ml. to tube 2. The maximum reaction produced by either of these dilutions, if greater than that obtained with undiluted serum, is reported as the result of the test.

Quantitative Serum Test. Quantitative tests are performed on serially diluted serum in saline, each dilution of which is treated as an individual serum and tested as described under the "Qualitative Serum Test." Freshly prepared 0.9 per cent saline is used for these dilutions. Serum dilutions are prepared by placing 0.5 ml. saline in each of six or more tubes; 0.5 ml. heated serum is added to tube 1, mixed well, and 0.5 ml. transferred to tube 2. This operation is continued until the sixth tube contains 1 ml. Dilutions of 1 : 2, 1 : 4, 1 : 8, 1 : 16, etc., are thus obtained.

Each serum dilution is tested and the greatest dilution yielding a *positive* (not *weakly positive*) reaction is reported as the reactivity end point in accordance with the following example:

Serum Dilutions						Report
1 : 2	1 : 4	1 : 8	1 : 16	1 : 32	1 : 64	
P	P	P	W.P.	N	N	Positive, 1 : 8 dilution
P	W.P.	N	N	N	N	Positive, 1 : 2 dilution
W.P.	N	N	N	N	N	*Positive, undiluted only

*Positive reaction obtained with undiluted serum

If other quantitative patterns are desired, serum dilutions of 1 : 5, 1 : 10, 1 : 20, etc., may be prepared, tested, and reported as above.

Cardiolipin Antigen in the Kolmer Complement-fixation Test

An antigen composed of 0.03 per cent cardiolipin, 0.05 per cent lecithin, and 0.3 per cent cholesterol in absolute alcohol may be substituted for Kolmer antigen in the Kolmer complement-fixation test for syphilis. The antigen is diluted 1 : 150 in saline and used in the test in exactly the same manner as Kolmer antigen. Cardiolipin antigen has been found to possess a minimal degree of nonspecific fixability (Harris, Portnoy, 1944) and it is probably not necessary to pretest complement to be used with it.

Interpretation

The incidence of positive reactions in the different stages of syphilis varies according to the sensitivity of the tests employed. The relative frequency of negative reactions in early primary syphilis must be kept in mind, and darkfield examinations should be utilized for diagnosis. Most patients show a positive serologic reaction within a month after the appearance of the chancre. The proportion of positive reactions to be expected in the other stages of syphilis is summarized:

Secondary syphilis	100%
Tertiary syphilis	60— 75%
Congenital syphilis	90—100%
Cerebrospinal syphilis	80— 95%
Paresis	90—100%
Tabes dorsalis	50— 75%

A positive serologic reaction may be obtained in the cerebrospinal fluid of patients whose blood gives doubtful or even negative reactions. A positive reaction may be obtained in the spinal fluid of patients with syphilis who show no clinical evidence of disease of the central nervous system. An examination of the spinal fluid should, therefore, be made in every patient having syphilis before treatment is terminated.

The various accepted serologic tests for syphilis are remarkably sensitive and specific, but it is a well-known fact that false positive reactions do occur. These may be classified as either technical or biologic.

Technical false positives may occur in any laboratory. The frequency of their occurrence is usually inversely proportional to the care exercised in the performance of the tests and rigid adherence to the prescribed technic. A single determination should never be considered as sufficient evidence for diagnosis. Repeated examinations should be made in the same or in another laboratory, and more than one type of test should be performed on the second specimen.

Positive serologic reactions are obtained in a large proportion of patients with yaws and pinta and are of great diagnostic value, but are of no assistance in differentiating between these three diseases.

Biologic false positives are associated with other diseases, such as leprosy, malaria, upper respiratory infections, infectious mononucleosis, and any febrile infection or immunization process. Many false positives are transitory in nature and become negative as the disease subsides and the patient improves. Quantitative determinations showing a progressive decrease in titer without treatment are strongly suggestive of false positive reactions. Retesting repeatedly at intervals of from 10 days to 3 weeks is at present the most practical method of studying reactions thought to be due to diseases other than syphilis. In the absence of significant history, clinical findings, or symptoms, it is well to wait for three to six months before making a final diagnosis. The majority of sera with false positive reactions become negative within three or four months. However, some individuals have been known to give such reactions for several years with no apparent cause.

It should also be recognized that false negative reactions may occur. A negative reaction is never more than presumptive evidence against the existence of syphilis. *Final interpretation of the result, whether positive or negative, rests with the clinician and always requires consideration of the history and clinical findings in each individual case.*

A syphilitic mother who has been treated during pregnancy but whose serologic reaction remains positive may give birth to a healthy uninfected infant whose blood yields a positive serologic reaction. In such cases the titer of the serum falls progressively and usually becomes negative within two months. Presumably there has been a passive transfer of reagin from the mother. If the titer increases or fails to fall, if it remains positive after two months of age, or if there are clinical manifestations of the disease, the reaction must be regarded as diagnostic and treatment instituted promptly.

The significance of a reaction which remains positive in spite of intensive treatment is not yet entirely clear. In many cases this is associated with serious lesions, particularly of the central nervous system, the aorta, or the viscera, and it is always an indication for an exhaustive examination. In other cases no such lesions can be found, and in them it does not necessarily convey an unfavorable prognosis or indicate the need for more than the usual full course of treatment.

CHAPTER 11

Preparation of Media and Reagents

Satisfactory media, reliably prepared, are essential to the maintenance of a good laboratory. About one-half of the personnel-time will be devoted to the making of media and the care of equipment.

Preparation of Glassware

All glassware coming from the laboratory, except syringes and pipets, should be placed in buckets, cans, or other suitable containers and autoclaved or boiled before handling. Used syringes and pipets can be put in cylinders or jars of cresol solution and then washed thoroughly in water. Particulate material is scraped from the glassware after autoclaving. Some articles can be cleaned immediately with soap, rinsed in tap water followed by distilled water, and dried in the air. Other pieces require more strenuous treatment, such as boiling in a detergent solution or soaking overnight in a cleaning mixture (p. 361), before washing. Pipets, after the plugs have been withdrawn, should be soaked in cleaning solution and rinsed thoroughly in water. Syringes must be cleaned meticulously and dried completely before packaging. Needles are best cleaned by forcing through them successively, water, alcohol, and ether. The outside surfaces can be polished with a mild abrasive and the points should be sharpened on a whetstone. Glassware that does not dry well in the open air can be placed in the hot-air oven for a short period; this is especially desirable for pipets, syringes, and small flasks.

Berkefeld filters are scrubbed with a brush, after being disinfected in weak cresol solution, and are rinsed by forcing water through in the reverse direction from that used to filter. They can then be boiled in a mild carbonate solution and rinsed again before packaging. Filters should not be heated (autoclaved) before they are cleaned. Seitz filters are disassembled, the pads discarded, and the filter washed thoroughly in cresol before rinsing and autoclaving.

Sterilization. Clean glassware must be packaged and sterilized, usually in the autoclave at 15 pounds pressure for 15 minutes. The dry oven is also satisfactory when employed at a temperature of 160° C. for 90 minutes. Packaging is done in such a manner as to assure continued sterility. Pipets are plugged with nonabsorbent cotton and wrapped individually in tissue paper or packaged without wrapping in metal pipet boxes. Petri dishes are wrapped in heavy paper, singly, in pairs, or in groups of five. The tops of beakers and flasks can be covered with paper which is secured with string. Culture tubes and small flasks are best plugged with nonabsorbent cotton. Syringes should be put up individually, either in cloth or paper wrappers or in large test tubes; syringes should always be autoclaved and packaged with the barrels removed from the jackets; and the numbers on barrel and jacket must correspond. Needles can be placed in the same packages with the syringes or handled separately in plugged serologic tubes. In the latter instance a generous plug of cotton should be placed in the bottom of each tube in order to protect the needle points.

Thorough familiarity with the autoclave and other items of equipment is essential for the laboratory worker. An excellent manual, *Sterilization,* by W. B. Underwood, can be obtained from the American Sterilizer Company of Erie, Pa. Effective sterilization in

the autoclave depends upon the *temperature* obtained, and not directly upon the pressure. The pressure-temperature relations shown in the table apply only if the sterilizer chamber is freed and kept free of air during the period in which steam enters the chamber. This can be accomplished by keeping the chamber drain valve slightly but continuously open. If air remains in the chamber, the temperature actually attained is much lower than that indicated, and sterilization will be entirely ineffective.

Table 39

PRESSURE-TEMPERATURE RELATIONS FOR THE AUTOCLAVE

Pounds	*Temp. C.*	*Pounds*	*Temp. C.*	*Pounds*	*Temp. C.*	*Pounds*	*Temp. C.*
1	102.3°	7	111.7°	13	119.1°	20	126.2°
2	104.2	8	113	14	120.2	22	128.1
3	105.7	9	114.3	15	121.3	24	129.3
4	107.3	10	115.6	16	122.4	26	131.5
5	108.8	11	116.8	17	123.3	28	133.1
6	110.3	12	118	18	124.3	30	134.6

The Arnold Sterilizer and special inspissators are unnecessary. An autoclave or steam-pressure sterilizer equipped with a thermometer and an air outlet near the bottom serves the same purposes.

Preparation of Media

Media are substances used for the cultivation of bacteria and related organisms. They are adapted to meet the peculiar growth requirements of the individual species and are prepared in several physical states—solids, semisolids, and liquids. The solid media contain gels, such as agar or gelatin, or ingredients which coagulate when heated, such as egg albumen and serum. The constituents of media serve such purposes as (*a*) providing substances for bacterial growth (carbohydrates, peptones, proteins, and "vitamins"), (*b*) indicators, (*c*) dyes and other substances which inhibit undesired forms, and (*d*) organic and inorganic salts necessary for growth, for the demonstration of biochemic activity, or for the maintenance of a constant pH.

Media can often be purchased in dehydrated form from such companies as the Difco Laboratories of Detroit, Mich., and the Baltimore Biological Laboratory of Baltimore, Md. Such media are uniform in behavior, easily prepared, and can be handled in small amounts. They are, in general, desirable for small facilities and are often economical even for large laboratories. Many are mentioned in this text.

Several procedures are applicable to media preparation in general. Most compounds must be cooked or heated to effect solution. This must be done in such a manner as to avoid undesirable changes in the ingredients. Temperatures of roughly 100° C. can be obtained by heating over water (double boiler or Arnold Sterilizer) or in the autoclave without pressure. Higher temperatures can be obtained, in media with a boiling point above that of water, by heating directly over a burner, stirring constantly to prevent burning. The addition of salts to the water in a double boiler also results in temperatures above 100° C. Many media can be cooked in the autoclave under pressure and the temperatures to be expected with various pressures are given above.

Often, it is necessary to clarify media at some stage in preparation. This can be done in a variety of ways and each problem must be considered individually. As a general rule strenuous methods should be avoided and only enough should be done to accomplish the desired end. Many of the liquids can be clarified by allowing them to stand until the particles settle out. Others can be passed through wire strainers, gauze, cotton, glass wool, or filter paper. Centrifuging will remove the particles from small amounts

media and the addition of egg albumen will often clarify hot solutions by the formation of a coagulum.

Reaction of Media. Adjustment of the pH of a medium is frequently necessary and is best done colorimetrically. Sealed colorimetric standards purchased from supply houses are more accurate and stable than those prepared in the laboratory. However, Table 41 is included for those desiring to prepare their own; it is only necessary to make the phosphate-hydroxide mixtures and add 10 drops of indicator to each 10 ml. of buffer. Table 40 gives the pH range for various common indicators as well as the desirable concentration for stock solution. Phenol red and bromthymol blue are most useful.

To make stock solutions of indicators, 0.1 Gm. indicator is weighed and ground, preferably in an agate mortar, with the volume of N/20 sodium hydroxide given in Table 40. When solution is complete, water is added to make a total volume of 25 ml.

For use the solution is diluted as noted in Table 41.

For the actual adjustment of media, a 2-ml. sample of the medium is placed in a tube the same size as that containing the standards. This is diluted to 10 ml. and 10 drops of indicator are added; the tubes are then placed in a comparator block. The usual adjustment necessary for media will be that of increasing the alkalinity and this is done by adding N/20 NaOH until the desired pH is attained (N/20 NaOH is 2 Gm. per liter). From the amount necessary to make the correction on 2 ml. of media, it can be calculated how much N/1 NaOH must be added to the entire lot.

Table 40

INDICATORS, THEIR pH RANGE, THE VOLUME OF N/20 NaOH NECESSARY TO EFFECT THE SOLUTION OF 0.1 GM., AND THE AMOUNT OF WATER USED TO DILUTE THE INDICATOR FOR USE

Indicator	*Ml. of N/20 NaOH*	*Test Solution: 1.0 ml. Indicator Plus ml. H_2O**	*pH Range*
Bromcresol purple	3.7	9	5.2 to 6.8
Bromthymol blue	3.2	9	6.0 to 7.6
Phenol red	5.7	19	6.8 to 8.4
Cresol red	5.3	19	7.2 to 8.8

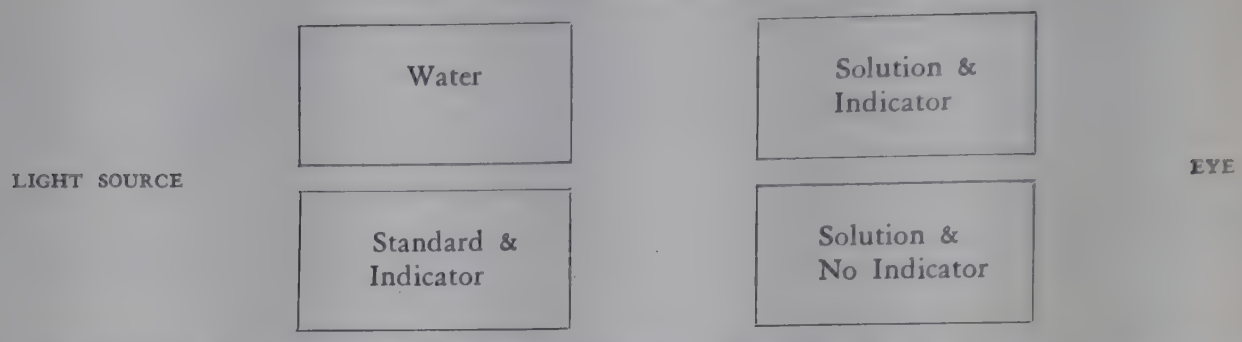
*Number of ml. of H_2O to be added to 1.0 ml. of indicator to make the test solution.

Table 41

GRADED MIXTURES OF KH_2PO_4—NaOH

pH	*Ml. of M/5 KH_2PO_4*	*Ml. of M/5 NaOH*	*Dilute to*
5.8	50	3.72	200 ml.
6.0	50	5.70	200 ml.
6.2	50	8.60	200 ml.
6.4	50	12.60	200 ml.
6.6	50	17.80	200 ml.
6.8	50	23.65	200 ml.
7.0	50	29.63	200 ml.
7.2	50	35.00	200 ml.
7.4	50	39.50	200 ml.
7.6	50	42.80	200 ml.
7.8	50	45.20	200 ml.
8.0	50	46.80	200 ml.

DIAGRAM OF COMPARATOR BLOCK



Sterilization and Storage. Media are sterilized by heat or by filtration. Whenever possible the autoclave should be used but occasionally certain substances will not stand the heat attained with steam under pressure. It is then necessary to use lower temperatures for a greater period of time. Fractional sterilization is often valuable, exposing the substance to moderate heat for 20 minutes on three consecutive days. The method to be used will be stated for each individual medium, and it will be seen that 15 or 20 minutes at 15 pounds autoclave pressure is the common rule. It is a good policy to avoid as much contamination as possible during the preparation of media even though thorough sterilization will minimize the effect of sloppy technic.

Media are best prepared frequently in small amounts so that the periods of storage are kept to a minimum. When large amounts are to be kept on hand they should be stoppered so as to prevent evaporation and usually kept in an icebox or cold room.

Finished media should be checked for sterility by incubating a representative sample overnight and examining it for growth. This is especially important for mixtures containing ingredients which have not been autoclaved, such as blood agar plates.

Preparation of Media for Neutralization of Bacteriostatic Drugs. Drugs, given a patient therapeutically, may interfere with the efforts of the laboratory. This may be minimized when it is known that a given fluid contains bacteriostatic drugs. Sulfonamides can be neutralized by the addition of 5 mg. per cent of para-amino-benzoic acid to the medium. A solution of the acid can be made up aseptically in such a concentration that this amount can be added when indicated. Penicillin can be neutralized by the addition of penicillinase (prepared by the Schenley Laboratories, New York). Ten units of penicillinase should be added to each 15 ml. of solid or liquid medium before inoculation. Penicillinase solutions and medium containing this enzyme are stable for at least three months if stored at 10° C.

Formulas for Individual Media

Meat Extract Broth (Basic Broth Medium): USE. This is a satisfactory broth for general laboratory purposes. It will support the growth of many common pathogens. This broth can be modified to make special-purpose media of a number of types.

INGREDIENTS:

Beef extract	3 Gm.
Tryptose (or proteose peptone #3)	20 Gm.
Sodium chloride	5 Gm.
Dextrose	1 Gm.
Distilled water	1000 ml.

PREPARATION. The ingredients are combined and dissolved with heat. The pH is adjusted to 7.4 to 7.5 and the preparation boiled for three minutes. It is then made up to volume, filtered through paper to clarify, dispensed, and autoclaved at 15 pounds for 20 minutes. Para-amino-benzoic acid can be added directly at this time or only when indicated.

A satisfactory agar medium can be made from this broth by the addition of 1.5 to 2.0 per cent agar.

Meat Infusion Broth and Agar: USE. This is superior to extract broth for routine use.

INGREDIENTS:

Lean meat (preferably beef)	500 Gm.
Neopeptone	10 Gm.
Sodium chloride	5 Gm.
Distilled water	1000 ml.

PREPARATION. The meat is ground, water added, and the mixture allowed to stand overnight in the icebox. Peptone and salt are added and the preparation heated to 65° C. for 30 minutes. It is then boiled for 10 minutes, strained through gauze, and filtered through paper, or better, allowed to settle until clear. It is made up to original volume and pH adjusted to 7.8. It is autoclaved at 20 pounds for 30 minutes and reclarified if necessary. The pH is readjusted to 7.4 to 7.6; the preparation is dispensed and autoclaved again for 15 minutes at 20 pounds.

If agar is desired, 2 per cent agar is added to the broth. It is heated to dissolve, clarified if necessary, and autoclaved.

Semisolid Agar: USE. This is excellent for primary isolation of many bacteria; it is especially good when micro-aerophilic forms are thought to be present.

INGREDIENTS AND PREPARATION. To extract broth, infusion broth, tryptose phosphate broth, or the combination base mentioned below (without the unlaked blood or agar) is added 0.1 to 0.3 per cent agar. The mixture is heated to dissolve the agar, clarified if necessary, and dispensed in large test tubes in amounts to allow a column of at least 6 cm. It is autoclaved at 15 pounds for 15 minutes.

To use, 1 to 2 ml. of a blood sample, or of spinal or other fluid, is added. The mixture is rotated to mix and incubated. Colonies will develop either in the medium itself or at the surface of the medium.

This medium is improved by the addition of 0.2 Gm. KCl and 0.1 Gm. $CaCl_2$ per liter.

Blood Agar: USE. This is the usual solid medium for the isolation of most common pathogens.

INGREDIENTS AND PREPARATION. To extract agar, infusion agar, or combination base agar is added 5 per cent defibrinated or citrated blood. The agar should be melted and cooled to 45° C. before adding the blood and, after mixing, pour plates or slants can be made. Horse or rabbit blood is best but human blood can be used when these other types are not available. Sheep blood is also satisfactory when other sources are not available. Precautions to maintain asepsis must be observed.

It is often advantageous, in making blood agar slants, to pour onto a solidified slant of basic agar enough of the freshly mixed blood agar to make a layer about 5 mm. thick. This is immediately slanted in proper position and allowed to solidify. Prepared in this way, the medium is more transparent, and the characteristics of surface colonies and evidence of hemolysis or discoloration can be seen almost as well as on a poured plate. It is also economical of blood.

With the addition of 1 per cent glycerol, blood agar is an excellent medium for the tubercle bacillus, and also for the organism of glanders.

Chocolate Agar: USE. Chocolate agar is used chiefly for cultivating members of the Hemophilus or Neisseria group.

INGREDIENTS AND PREPARATION. The contents of tubes of extract, infusion, or combination agar base are melted. They are then cooled to 55° C. and 5 per cent blood is added. The temperature is raised slowly to 85° C. and the mixture allowed to cool to 45° C. It is dispensed as pour plates or slants.

Combination Agar: USE. This is utilized in an attempt to put into one medium growth factors for as many pathogens as possible. This medium will support both the gonococcus

and the Hemophilus as well as Brucella. It may be used routinely for the study of material containing mixed or unknown bacteria.

INGREDIENTS:

Beef extract	3 Gm.
Tryptose	10 Gm.
Proteose peptone #3	10 Gm.
Sodium chloride	5 Gm.
Dextrose	0.3 Gm.
Nicotinamide agar 15 Gm.	0.1 Gm.
Distilled water	1000 ml.
Blood	100 ml.

PREPARATION. The ingredients other than blood are combined and heated until the solids are dissolved. The pH is adjusted to 7.4 to 7.5 and the mixture boiled for a few minutes. It is filtered through paper if necessary and dispensed for storage. When desired for use, this base is melted and when the temperature is 90° C. 5 per cent blood is added. While still hot the preparation is centrifuged to remove coagulum, then cooled to 45° C., 5 per cent more blood is added, and the medium poured as plates or slants. The chocolating process can be eliminated by adding 0.1 per cent laked red cells along with the 5 per cent fresh blood. This medium without agar and without the fresh blood makes an excellent liquid base. It is autoclaved at 15 lbs. for 20 minutes.

Fluid Thioglycollate Medium: USE. This is a good medium for general use and for sterility testing. It will support a growth of Clostridia.

INGREDIENTS:

l-cystine	0.75 Gm.
Sodium chloride	2.5 Gm.
Glucose	5.0 Gm.
Agar	0.75 Gm.
Water-soluble extract of yeast	5.0 Gm.
Pancreatic digest of casein (peptone)	15.0 Gm.
Sodium thioglycollate	0.5 Gm.
Thioglycollic acid	0.3 Gm.
Resazurin, 0.1% fresh solution	1.0 ml.
Distilled water	1000.0 ml.

PREPARATION. Dry ingredients, except the thioglycollate, are mixed in a mortar in the order given above. Then a portion of the water, heated, is added with stirring, and the whole material transferred to a flask, the remainder of the water added, and solution completed in a steam bath. Then the thioglycollate is added, the pH adjusted with NaOH, and finally the resazurin added. The medium is dispensed in test tubes and autoclaved for 18 minutes at 120° C.

Phenol Red Broth: USE. This is a base to which test carbohydrates can be added for the study of fermentative activity. This medium will support the growth of Neisseria and Corynebacterium as well as members of the coliform group.

INGREDIENTS:

Beef extract	3 Gm.
Proteose peptone #3	10 Gm.
Sodium chloride	5 Gm.
Phenol red (Bacto)	0.018 Gm.
Distilled water	1000 ml.

PREPARATION. The ingredients are dissolved with heat, the pH adjusted to 7.4, the mixture dispensed and autoclaved at 15 pounds for 15 minutes. Most carbohydrates should

be made up in a 10 or 20 per cent aqueous solution and sterilized by passage through a Berkefeld filter. When such equipment is not available, the sugar solution is autoclaved separately for 10 minutes at 12 pounds pressure. Enough of the carbohydrate solution is added to give 0.5 per cent, and the medium dispensed aseptically into tubes.

The Dunham type of fermentation tube is good for the study of groups producing gas during growth. These tubes are made by placing small inverted tubes in 150- × 15-mm. tubes. These small inverted tubes can be made by sealing one end of 1½-inch pieces of 6- to 8-mm. glass tubing. Fermentation reactions of the Neisseria are studied by adding 0.15 per cent agar or by using a solid slanted medium with carbohydrate and indicator.

Other satisfactory fermentation bases can be improvised as needed. It is only necessary to have a sugar-free medium to which can be added an indicator and the test carbohydrate; the nutrient broth for water analysis can be used for members of the coliform group. Infusions cannot be used unless the carbohydrate from the meat is first removed by preliminary fermentation with colon bacilli, which can then be killed by heat and removed by centrifuging or filtration. Phenol red base broth can be purchased in dehydrated form from Difco.

Enlow's Medium: Use. This is a simple, sugar-free, semisolid agar medium for studying fermentation of most ordinary bacteria. Acid is shown by change in color of indicator; gas, by bubbles in the semisolid medium.

Ingredients:

Tap (or distilled) water	1800.00 ml.
Peptone	10.0 Gm.
Sodium phosphate (Na_2HPO_4)	14.5 Gm.
Potassium phosphate (KH_2PO_4)	1.4 Gm.
Agar	2.0 Gm.

Preparation. These substances are heated together, filtered through paper while hot, and diluted with hot distilled water to 2000.00 ml.

Carbohydrates are added in 0.5 per cent amounts. Bromthymol blue indicator is added to give a distinct blue-green color.

The medium is then tubed and sterilized.

Carbohydrates that are easily hydrolyzed should be sterilized separately in aqueous solution and added to the bulk medium, which is then tubed aseptically.

The finished medium has a pH of 7.2 to 7.4.

Bacteria of the genera Neisseria and Hemophilus, and other delicate microörganisms will not grow well on this simple medium, but prefer one of the fermentation bases described under phenol red broth.

Proteose Peptone #3 Chocolate Agar: Use. An easily prepared medium for cultivation of the gonococcus.

Ingredients:

Proteose #3 agar (Difco)	9 Gm.
Bacto-hemoglobin	2 Gm.
Water	200 ml.

Preparation. The agar is dissolved in 100 ml. water by heating gently. This solution is autoclaved for 15 minutes at 15 pounds pressure. The hemoglobin is dissolved in the remainder of the water which should be brought to about 50° C. Any particles are removed by passage through moist cheesecloth or gauze. This solution is autoclaved for 20 minutes at 15 pounds pressure. Both solutions are cooled to 50° C., mixed, and pour plates made. Bacto-supplement "A," a yeast extract obtainable from Difco, enhances the efficiency of this medium. Crystal violet in concentrations of 1 : 700,000 or tyrothricin to a concentration of 1 : 15,000, will inhibit the growth of Gram-positive contaminants.

Mueller's Starch-casein Hydrolysate Medium: Use. This medium is used for isolation of the Neisseria, especially when satisfactory blood-containing medium is unavailable.

Preparation and Ingredients. Seventeen Gm. dry, shredded agar are dissolved in 500 ml. tap water by autoclaving for 15 minutes at 15 pounds pressure.

While this solution is still hot the following are added:

Meat infusion	300.0 ml.
Casein hydrolysate (Difco)	17.5 Gm.
Starch paste*	100.0 ml.
Water	100.0 ml.

The pH is adjusted to 7.4 to 7.6. The solution is mixed and distributed into tubes or flasks. It is autoclaved not more than 10 minutes at 10 pounds pressure.

This medium may be used for plating or as slants. It is especially useful when made up as a semisolid medium by using less agar.

Levinthal Medium: Use. *Hemophilus influenzae* will grow on this medium.

Preparation and Ingredients. Any good meat infusion broth base may be used such as that described on p. 341. To this is added 0.03 per cent glucose and 10 per cent blood (sheep or rabbit). The mixture is placed in a cool water bath. The temperature is then raised to 100° C. and held at this point for three to five minutes. At the end of this time the blood is coagulated and of a uniform chocolate color. The brown precipitate is then removed by filtration through paper, and the medium is immediately sterilized by filtration through a Berkefeld N candle.

For a solid medium one part of this broth is added to one part of a 4 per cent nutrient agar base. The agar is melted and partially cooled before the broth is added. Plates are used on the day that they are poured.

Fildes Peptic Digest: Use. This digest supplies accessory factors for the growth of *Hemophilus influenzae*.

Preparation and Ingredients. Into a 250-ml. glass-stoppered bottle are placed the following:

Physiological salt solution	150.00 ml.
Hydrochloric acid, concentrated	6.00 ml.
Defibrinated sheep or rabbit blood	50.00 ml.
Granulated pepsin	1.0 Gm.

The bottle is shaken to dissolve the contents. After incubation at 55° C. for 2 to 24 hours, 12 ml. of 20 per cent NaOH are added. The reaction is tested with cresol red, then HCl is added drop by drop until the pH equals 7.0 to 7.2, and 0.25 per cent chloroform is added. The acid causes a precipitate of hematin.

For use, the digest, in the proportion of 2 per cent, is added to a good nutrient broth medium; or, in the proportion of 2 to 5 per cent, to a nutrient agar.

Bordet-Gengou Medium (Modified): Use. This medium is used for the isolation of *H. pertussis*. The tubercle bacillus grows well on it also.

Ingredients:

Potatoes, peeled and sliced	100 Gm.
Glycerin	8 ml.
Water	200 ml.
Agar	
Sodium chloride (0.75 per cent solution)	
Fresh blood	

*Starch paste is made by suspending 1.5 Gm. of either corn or laundry starch (not "soluble starch") in 10 ml. cold water. This is poured slowly into 90 ml. boiling water while stirring.

PREPARATION. The first three ingredients are mixed and then steamed in an autoclave or boiled until soft. The original volume is restored; the solution is strained through gauze and allowed to stand until sedimentation is complete. The clear broth is syphoned off and to each 50 ml. of this are added 150 ml. of 0.75 per cent saline and 5 Gm. agar. The mixture is heated to dissolve the agar, dispensed, and autoclaved for 25 minutes at 15 pounds pressure. When plates are desired, this base is melted and cooled to 45° C. From 10 to 15 ml. fresh blood are added to each 50 ml. base, and the mixture poured, allowing half again as much medium per plate as for the usual blood plate.

A satisfactory potato-glycerin base can be purchased in dehydrated form from Difco. It is necessary only to add fresh blood for use.

Loeffler's Serum Medium: USE. This medium is used primarily for the cultivation of the diphtheria bacillus. It can also be used for accentuating the color production of staphylococci or pigmented Neisseria and for testing proteolytic power.

INGREDIENTS:

Extract broth (p. 340)	50 ml.
Beef, horse, hog, or human serum	150 ml.
Dextrose	0.450 Gm.

PREPARATION. Dextrose is added to the extract broth and the pH adjusted to 8.0 with NaOH. The fresh serum is added and mixed. The medium is dispensed in 3- to 5-ml. amounts in culture tubes (150 × 15 mm. or similar size). The tubes are slanted. They are inspissated and sterilized as follows: They are placed in a slanting position in the autoclave and covered with paper to avoid sudden contact with steam and to protect them from condensation water. The door and air outlet valve of the autoclave are closed. The pressure is gradually raised to 15 pounds, without letting any of the air escape. The pressure is maintained for at least 10 minutes, or until the temperature reaches at least 100° C. The air-outlet valve is opened so slightly that the pressure will not vary more than ½ pound—thus allowing the condensed water and some of the confined air to escape. The valve is closed and the sterilization process continued for 20 minutes at 15 pounds pressure. After sterilization is completed, the source of steam is cut off and the autoclave allowed to cool slowly until the pressure is nil. The final reaction of the medium (the water of syneresis may be tested) should be between pH 7.6 and 7.8. Alternative methods of inspissation and sterilization include: (*a*) inspissation in a double boiler until coagulation is complete and then sterilization in the Arnold Sterilizer for 20 minutes on three successive days; (*b*) inspissation in the dry oven at 85° C. and then sterilization in the autoclave without pressure or with pressure not in excess of 7 pounds. The finished product should be free from bubbles and there should be two or three drops of condensation water at the butt of the slant.

It is essential that this medium be fresh and moist; small amounts should be made frequently and the period of storage kept at a minimum. It is often more convenient to obtain the dehydrated product, such as is supplied by the Difco Laboratories.

Cystine Tellurite Agar: USE. This is used for the cultivation and differentiation of members of the genus Corynebacterium.

INGREDIENTS:

Infusion or extract agar	15 ml.
Blood or serum	0.75 ml.
0.3% potassium tellurite	2.25 ml.
Cystine	few granules

PREPARATION. The agar is melted and cooled to 45° C. The other ingredients are added and the mixture poured into a Petri dish. When larger amounts are prepared the cystine is added in 0.05-Gm. amounts per liter of agar. The tellurite solution must be titrated

since various lots differ considerably. The product, as prepared by A. H. Thomas Company, Philadelphia, bears a titration figure and instructions for use. A dehydrated blood-tellurite mixture can be purchased from the Difco Laboratories and added to a basic agar medium, resulting in a satisfactory product.

Glucose-Cystine-Blood Agar: USE. This agar is used for the isolation of *Pasteurella tularensis.*

INGREDIENTS:

Beef infusion agar	100 ml.
Cystine	0.10 Gm.
Glucose	1.0 Gm.
Blood (rabbit preferred)	8.0 ml.

PREPARATION. The agar is melted in steam or water bath; glucose and cystine are added and heating is continued for 20 minutes. After cooling to 50° C., blood is added and the mixture heated over boiling water for two hours. It is then cooled, dispensed, and incubated for sterility.

The Difco Laboratories' "Cystine-Heart Agar" with fresh rabbit blood can be substituted for this medium.

Avery Broth for Pneumococcus: USE. This is recommended for the rapid proliferation of pneumococci when mice are not available. Sterile ascitic fluid can also be used.

INGREDIENTS:

Infusion broth	90 ml.
Glucose	1 Gm.
Blood (rabbit or horse)	5 ml.

PREPARATION. The pH of the broth is checked and adjusted to 7.6 to 7.8. Glucose, preferably as a sterile 20 per cent solution (5 ml.), and blood are added. The medium is then distributed, allowing 5 ml. per tube.

Tryptose Broth and Tryptose Agar: USE. It is generally agreed that these media are preferable to liver infusion for the isolation, growth, and study of the Brucella. Both can be obtained in dehydrated form from the Difco Laboratories, or the agar can be prepared from the broth by the addition of 0.1 per cent dextrose and 1.5 per cent agar.

INGREDIENTS:

Tryptose	20 Gm.
Sodium chloride	5 Gm.
Distilled water	1000 ml.

PREPARATION. The ingredients are mixed and the solids dissolved by heat. After being dispensed the medium is autoclaved for 20 minutes at 15 pounds pressure. The final pH for Brucella should be 6.6 to 6.8.

Liver infusion and agar, using 500 Gm. liver for 1000 ml. media, can be substituted for tryptose broth and agar. It is made essentially as was the meat infusion, but does not give as constant results as the commercial alternative. Combination agar also supports the growth of Brucella.

Crystal violet agar, made by adding 1 ml. of a 1 : 700 solution of crystal violet dye to a liter of media, will suppress the growth of a number of the Gram-positive forms when it is necessary to isolate the Brucella from contaminated material. Basic fuchsin and thionin, used to differentiate the types of Brucella, can be prepared by adding 1.0 per cent aqueous solutions of the dye to melted tryptose agar. Both dyes can be sterilized by heating in boiling water for 15 minutes. Four ml. of the basic fuchsin are used per liter of medium or 2 ml. of the thionin. The same dyes can be incorporated into liver infusion agar or combination agar. Tryptose agar slants can be used for the determina-

tion of hydrogen sulfide production (see p. 351), but better results are obtained when liver or meat infusion (250 Gm. of meat per liter) is used instead of distilled water in the preparation of the slants.

Cooked-meat Tubes: USE. These are used for the propagation and study of anaerobes, especially of the Clostridia.

INGREDIENTS AND PREPARATION. To a tube of extract broth is added enough lean, ground beef or veal to occupy one-half the column of liquid. This is autoclaved for 30 minutes at 15 pounds. The pH is checked and adjusted to 7.2 to 7.6.

Brain Medium: USE. Used for determining proteolysis by spore-forming anaerobes. The proteolytic species cause a blackening of the medium.

INGREDIENTS AND PREPARATION. Sheep or beef brains are boiled with an equal amount of water. The water is decanted and the brain passed through a colander. Peptone, 2 per cent, and dextrose, 0.1 per cent, are added to the decanted water and the reaction made slightly alkaline; i.e., pH = 7.2 to 7.4. Large test tubes are filled about one-half. These are sterilized in flowing steam for 30 minutes on each of 5 days, being held at room temperature during the intervals.

Whole-milk Tubes: USE. These are used for the cultivation of anaerobes.

INGREDIENTS AND PREPARATION. Fresh milk is shaken sufficiently to distribute cream and 7-ml. amounts are dispensed into test tubes. This is autoclaved at 12 pounds for 10 minutes. Before inoculating, it is reheated in a boiling water bath for 10 minutes, in order to drive out oxygen.

Bromcresol Purple Milk: USE. This is used for the study of acid production and clot formation in milk.

INGREDIENTS AND PREPARATION. Milk is skimmed free from cream and heated in boiling water or flowing steam for 20 minutes. Any fat forming on the surface is removed. Forty ml. of 0.04 per cent aqueous bromcresol purple (or 1 ml. of 1.6 per cent in 95 per cent alcohol) per liter of milk are added. The mixture is tubed in 5- to 10-ml. quantities, and sterilized for 20 minutes in steam or boiling water on three successive days. It is incubated for sterility before use.

Special Media for the Tubercle Bacillus: N SOLUTION. It is unnecessary to add peptone or meat infusion to media containing blood, egg, or potato base. Ammonium salts or asparagin furnish a readily available source of nitrogen. Five parts of ammonium citrate, five parts of asparagin, and one part of magnesium sulfate are ground up thoroughly together; dissolved in 1-Gm. lots in 100 ml. water and sterilized in screw-capped bottles.

EGG-YOLK MEDIUM EMPLOYING N SOLUTION. Aseptic precautions should be observed in preparing the medium as it is difficult to sterilize. The necessary number of clean fresh eggs are immersed in 70 per cent alcohol for 10 minutes. With scrubbed hands and sterile rubber gloves, the eggs are broken and the yolks separated and allowed to fall (and break) into a sterile flask containing glass beads and marked to indicate the volume of yolk required. The flask is shaken to disperse the yolks. To each 67 ml. yolk, 3 ml. sterile glycerin and 30 ml. sterile N solution are added and the flask again shaken. The mixture is then poured into a previously sterilized funnel fitted with a layer of gauze and a pinch clamp. It is tubed in amounts sufficient to make long slants or to cover the side of screw-capped bottles to a depth of at least 5 mm. It is sterilized by inspissation for two hours on two successive days. It is possible to sterilize in the autoclave if special precautions are taken to avoid disruption by air bubbles.

The addition of 1 : 1000 Congo red makes early colonies more easily visible. Contaminations may be reduced by adding 1 : 1000 malachite green (equivalent to Sonnenschein's medium) or 1 : 10,000 gentian violet (equivalent to Petroff's medium).

LÖWENSTEIN'S MEDIUM. In the preparation of this medium aseptic precautions are observed as for egg-yolk medium described above. To 100 ml. of N solution 4 Gm. potato flour and 12 ml. glycerin (2 ml. if bovine bacilli are anticipated) are added. The mixture is stirred, boiled for 15 minutes, and cooled. It is then added to 100 ml. whole egg,

well shaken. After mixing and filtering, the medium is tubed and sterilized in the same manner as the egg-yolk medium described above.

These media may be preserved for four months in air-tight, screw-cap vials or bottles.

Inspissation and Sterilization. To avoid disruption of the medium by air bubbles the following steps are taken:

The freshly tubed medium is placed in the icebox overnight, and then heated to a temperature below the coagulation point, until the air is expelled; or the tubes are placed in a large vacuum desiccator for an hour, after evacuating the air.

The tubes are then placed in a slanting rack or similar support and heated in a *tightly sealed* autoclave. When the pressure reaches 10 pounds the air valve is opened just enough to permit some exchange of air for steam without altering the pressure. When the thermometer indicates that the corresponding temperature has been attained, the air valve may be closed and sterilizing continued for 10 minutes, with the heat or steam flow reduced so that the *same* pressure is maintained. The air valve should be left closed until the pressure has fallen to atmospheric level.

Egg-yolk Medium: Use. This is used for the cultivation of the tubercle bacillus as well as for *Pasteurella tularensis*.

Ingredients:

Eggs	4
Saline	30 ml.
Malachite green (2% aqueous)	5 ml.
Glycerin	see below

Preparation. Sterile equipment is used throughout and all possible sources of contamination are avoided since the finished medium is difficult to sterilize. The eggs are washed in soap and water, rinsed, and immersed in alcohol. The alcohol is removed by igniting. The eggs are opened; the yolks are placed in a beaker and the whites are discarded. To the yolks are added 30 ml. normal saline solution or, if the medium is to be used for the growth of human strains, 25.5 ml. saline and 4.5 ml. glycerin are used. After mixing thoroughly and filtering through a wire sieve, 5- to 6-ml. amounts are tubed, slanted, and sterilized as described for Loeffler slants (p. 345). This medium can be stored in the icebox for considerable periods if stoppered with rubber or paraffin.

Petragnani's Medium: Use. This medium is used for cultivation of the tubercle bacillus.

Ingredients:

Skimmed milk	450 ml.
Potato flour	18 Gm.
Asparagin	2.6 Gm.
Peeled and thinly sliced potatoes	225 Gm.
Eggs	12
Egg yolks	3
Sterile C.P. glycerin	35 ml.
2% aqueous malachite green (certified)	30 ml.

Preparation. The sliced potatoes, potato flour, milk, and asparagin are placed in a double boiler and cooked for two hours. The mixture is stirred constantly until it becomes sticky, after which occasional stirring will suffice. The eggs are sterilized by rubbing with a sponge soaked in 70 per cent alcohol. The egg yolks and egg white with the three extra yolks are dropped into a sterile liter Erlenmeyer flask. The egg yolks are broken with a sterile glass rod and then shaken well. A sterile rubber stopper is placed on the flask while the contents are shaken. The glycerin and malachite green are now added and mixed by shaking. The potato-milk mixture is cooled to 45° to 50° C. and the egg-glycerin mixture added slowly and mixed well. The medium is then filtered through sterile gauze into a sterile beaker. It should be neutral to litmus, pH = 7.0.

The medium is distributed aseptically in sterile test tubes and carefully inspissated. This may be done in the autoclave using the following procedure: The temperature is raised to 45° C. in the first half hour, to 65° C. in the second half hour, and to 80° C. in the third half hour. It is held at 80° C. for 20 minutes. The medium is then allowed to cool and sterile rubber stoppers are substituted for the cotton plugs.

An alternate autoclave method for coagulation and sterilization is that described for the preparation of Loeffler's serum slants (p. 345).

Kirchner's Medium: USE. This is a synthetic fluid medium for cultivation of the tubercle bacillus. It is especially valuable in preparation of purified tuberculin.

INGREDIENTS:

Sodium phosphate (Na_2HPO_4)	3.0 Gm.
Potassium phosphate (KH_2PO_4)	4.0 Gm.
Magnesium sulfate	0.6 Gm.
Sodium citrate	2.5 Gm.
Asparagin	5.0 Gm.
Glycerol	20.0 ml.
Water to	1000.0 ml.

PREPARATION. The ingredients are mixed and 2 ml. of 40 per cent NaOH are added to bring the pH to 7.2 before autoclaving.

Nutrient Gelatin: USE. This is used to demonstrate the ability of certain organisms to liquefy gelatin. It can also be used in place of agar for plates to be incubated at temperatures not above 20° C.

INGREDIENTS:

Beef extract	3 Gm.
Peptone	5 Gm.
Gelatin	120 Gm.
Distilled water	1000 ml.

PREPARATION. The ingredients are combined and the solids dissolved at 65° C. The solution is made up to volume and the pH adjusted to 7.0. The mixture is heated to boiling while stirring vigorously. It is again made up to volume, filtered through cotton if necessary, dispensed, and autoclaved for 20 minutes at 15 pounds pressure.

Sodium Hippurate Broth: USE. This is used for the differentiation of group B streptococci from the human strains.

INGREDIENTS AND PREPARATION. To extract broth is added 1 per cent sodium hippurate and the mixture tubed in 5-ml. amounts. The level is marked with a glass-marking pencil and the medium autoclaved for 15 minutes at 15 pounds pressure. The tubes are inoculated and the organisms allowed to grow in the incubator for 48 hours. At the end of this time enough distilled water is added to bring the column up to the original level. The culture is centrifuged. A reagent is prepared by adding 12 Gm. ferric chloride ($FeCl_3 \cdot 6H_2O$) to 100 ml. aqueous 2 per cent hydrochloric acid. Eight-tenths ml. supernatant fluid from the culture and 0.2 ml. reagent are mixed in a small tube. A precipitate present at the end of 15 minutes indicates a positive reaction (the formation of benzoic acid from sodium hippurate).

Citrate Agar: USE. Citrate agar is used for the differentiation of coli and aerogenes bacilli.

INGREDIENTS:

Sodium citrate	2 Gm.
Sodium chloride	5 Gm.
Magnesium sulfate	0.2 Gm.

Ammonium dihydrogen phosphate	1 Gm.
Dipotassium phosphate	1 Gm.
Agar	20 Gm.
Distilled water	1000 ml.

PREPARATION. The ingredients are mixed and the solids dissolved by heating. After the pH is adjusted to 6.8, 5 ml. of 0.5 per cent alcoholic bromthymol blue are added. The medium is dispensed, slanted, and autoclaved at 15 pounds pressure for 15 minutes. Satisfactory dehydrated citrate agar can be obtained from Difco Laboratories. A positive reaction (organism capable of existing with citrate as only source of nutrition) on this medium is characterized by the appearance of colonies and a change in the indicator from green to dark blue.

Tartrate Medium: USE. This medium is used in the differentiation of members of the genus Salmonella.

INGREDIENTS:

Sodium potassium tartrate	10 Gm.
Peptone	10 Gm.
Sodium chloride	5 Gm.
Agar	20 Gm.
Phenol red (0.2% alcoholic solution)	12 ml.
Distilled water	1000 ml.

PREPARATION. The ingredients are mixed and the solids melted in flowing steam or over boiling water. The pH is adjusted to 7.6 to 7.8. The medium is dispensed in 5-ml. amounts into test tubes and autoclaved for 20 minutes at 15 pounds. This medium may be obtained in dehydrated form from the Difco Laboratories.

Tubes of this medium are inoculated by stab and the reaction observed after 48 hours of incubation. Typical findings include the following:

ACID REACTION (YELLOW)	ALKALINE REACTION (RED)
Salmonella typhimurium	*Salmonella paratyphi B*
Salmonella enteritidis	*Salmonella paratyphi A*
Salmonella suipestifer	*Alkaligenes faecalis*
Salmonella typhi	
Proteus vulgaris	
Escherichia coli	

Peptone-dextrose Solution: USE. This solution is used for performing the Voges-Proskauer and methyl red tests in the differentiation of coli and aerogenes bacilli.

INGREDIENTS:

Proteose peptone (or Witte's peptone)	5 Gm.
Dextrose	5 Gm.
Dipotassium hydrogen phosphate	5 Gm.
Distilled water	1000 ml.

PREPARATION. The first three substances are mixed with 800 ml. water and dissolved with heat. The solution is cooled to room temperature and the volume brought up to 1000 ml. The solution is filtered through paper and dispensed in 10-ml. amounts into test tubes. Intermittent sterilization for 20 minutes every three days without pressure is recommended. A satisfactory commercial substitute is the Buffered peptone-dextrose (M-R, V-P, medium) of the Difco Laboratories.

The methyl red test is done after a three- or four-day growth is obtained. An indicator

is made by dissolving 0.1 Gm. methyl red in 300 ml. alcohol and bringing the volume up to 500 ml. with distilled water. Five drops of this indicator are added to 5 ml. of the broth and the color reaction recorded; a red reaction is positive, a yellow negative, an intermediate questionable.

The Voges-Proskauer test can be made on the same tube of broth by pipetting off 5 ml. after 48 hours of growth. To this is added 5 ml. of 10 per cent potassium hydroxide. The mixture is placed in the incubator. The development of an eosin hue within six hours indicates the presence of acetyl methyl carbinol and constitutes a positive reaction. The six-hour period of incubation can be reduced to one hour if 2 drops of 2 per cent ferric chloride are added before the potassium hydroxide.

Urea Medium: Use. Urea medium is used to determine the ability of organisms to change urea to ammonia. It is valuable in identification of the Proteus group of bacteria.

Ingredients:

Urea	2 Gm.
Yeast extract (Difco)	0.1 Gm.
M/15 primary phosphate buffer	500 ml.
M/15 secondary phosphate buffer	500 ml.

Preparation. To make the primary buffer, 4.535 Gm. monobasic potassium phosphate are added to 500 ml. water. For the secondary buffer, 4.735 Gm. dibasic sodium phosphate are added to 500 ml. water. All the ingredients are mixed and sterilized by filtration through a Seitz or a Berkefeld filter. The medium is dispensed in 2-ml. amounts in sterile tubes. The final pH will be about 6.8.

The culture tubes are inoculated and incubated at 37° C. After a 24- to 48-hour growth is obtained a few drops of Nessler's solution are added. The appearance of a yellowish-brown precipitate indicates that urea has been split. When Nessler's reagent is not available, the pH of the culture is tested; alkalinization indicates ammonia formation from urea.

Lead Acetate Agar: Use. Lead acetate agar is used for the detection of hydrogen-sulfide production by bacteria.

Ingredients and Preparation. Any satisfactory agar base including extract agar, infusion agar, or combination agar without blood may be used. The agar is melted and cooled to 50° C. One ml. of autoclaved 0.5 per cent aqueous basic lead acetate per 10 ml. of agar is added to the agar base. The medium is placed in culture tubes, allowed to solidify, and is inoculated by stab around the edges. Black discoloration after 24-hour incubation is indicative of sulfide production.

This method has been largely replaced by lead-acetate paper. Strips of filter paper are immersed in a sterile (boiling) 20 per cent solution of normal lead acetate. They are then dried and stored in sterile test tubes until needed. These strips can be inserted between the plug and wall of culture tubes so that an inch or two of the paper is exposed to the atmosphere over the medium. They must not be immersed in the culture broth or allowed to touch the slant. A positive result is obtained when the paper becomes blackened. The test can be roughly quantitative if the amount of blackening is observed or the paper changed frequently. Either solid or liquid medium can be used and the constitution of the medium is not very important except when testing strains of Brucella; here, liver infusion is essential for clear-cut results (p. 110). When determining production of hydrogen sulfide by Brucella, the papers are changed at the end of each 24 hours of incubation and the results interpreted as follows:

B. melitensis . . . . none or only a trace of blackening during four-day incubation.
B. abortus . . . . . moderate to marked blackening during the first two days of incubation.
B. suis . . . . . moderate to marked blackening throughout four days of incubation.

Peptone Water: Use. Peptone water is used for the demonstration of indol production.

Ingredients:

Tryptone (Difco) (or other suitable peptone)	10 Gm.
Sodium chloride	5 Gm.
Distilled water	1000 ml.

Preparation. The ingredients are mixed and the solids dissolved with heat if necessary. The solution is tubed in 5-ml. amounts, and autoclaved at 15 pounds for 15 minutes. The pH is checked; it should be around 7.3.

Cultures are grown in peptone water for 48 hours and at the end of this period, 1 ml. ether is added; the mixture is shaken and allowed to separate. A few drops of para-dimethylaminobenzaldehyde reagent is then poured down the side of the tube so that a ring is formed at the junction of the ether and culture fluid. A pink color at this interface denotes a positive reaction. The reagent is made by dissolving 2 Gm. aldehyde in 190 ml. ethyl alcohol and, after solution is effected, adding 40 ml. concentrated hydrochloric acid. The stock reagent should be stored in a dark bottle and out of the light.

A simpler method for determining indol production is to soak strips of filter paper in a saturated aqueous solution of oxalic acid, dry them, and use by suspending over the culture medium as was described for lead-acetate paper (p. 351). The development of a pink color on the paper denotes a positive reaction. The peptone in the medium must contain tryptophane which is the source of the indol.

Nitrate-peptone Water: Use. This is used for the determination of the formation of nitrites from nitrates.

Ingredients and Preparation. To the peptone water described above is added 0.1 per cent potassium nitrate. The mixture can be autoclaved to sterilize. It is important that water free from ammonia be used, and that the nitrate be nitrite-free. Nitrate-peptone water will not grow Hemophilus so that for these organisms it is necessary to add 2 per cent blood to the peptone water. This mixture is heated slowly to 80° C., cooled, and the coagulum removed either by filtering or centrifuging. The product, known as "hemo-peptone water," is sterilized by passage through a Berkefeld filter. The nitrate can be added to it before filtration, using enough to give a 0.02 per cent solution.

To determine whether nitrates have been reduced by bacterial growth, two reagents are prepared as follows:

Solution A:

Sulfanilic acid	0.8 Gm.
Sulfuric acid	5 ml.
Distilled water	95 ml.

Solution B:

Dimethylalphanaphthylamine	0.6 ml.
Glacial acetic acid	30 ml.
Distilled water	70 ml.

To 5 ml. of the nitrate-peptone water (or hemopeptone-nitrate water) is added 0.1 ml. of solution A. Then a few drops of solution B are added. In the presence of nitrites a red or pink color will develop. The reagents for this test are available in laboratories performing chemical analysis of water.

Tetrathionate Broth: Use. This is used as an enrichment broth for Salmonella; it allows these to proliferate while suppressing the growth of the lactose fermenting forms.

Ingredients:

Proteose peptone	5 Gm.
Bile salts	1 Gm.

Calcium carbonate	10 Gm.
Sodium thiosulfate	30 Gm.
Distilled water	1000 ml.
(Iodide solution)	see below

PREPARATION. The tetrathionate base is best purchased (Difco) in dry form and prepared for use as desired. To 100 ml. water are added 4.6 Gm. of base and the mixture brought to a boil. It is cooled to 45° C. and tubed in 10-ml. amounts. At the time of use, to each tube is added 0.2 ml. potassium iodide in 20 ml. water. Both the base broth and the iodide solution are stable but the two should not be combined until the time for use.

Selenite F enrichment medium is comparable to the tetrathionate broth in behavior and can be substituted if desired. It is obtainable in dehydrated form from the Baltimore Biological Laboratory. The formula is as follows:

Sodium acid selenite (anhydrous)	4 Gm.
Peptone	5 Gm.
Sodium phosphates (anhydrous)	10 Gm.
Distilled water	1000 Gm.

The relative amounts of monobasic and dibasic sodium phosphates that will give a pH of 7.0 are determined by trial. The weighed ingredients are dissolved in the distilled water, the solution tubed in 10-ml. amounts, and sterilized in flowing steam for about 30 minutes.

Eosin-methylene Blue Agar: USE. The agar is used to differentiate non-lactose-splitting colonies from the coli-aerogenes group. It is interchangeable with Endo's medium and MacConkey's medium.

INGREDIENTS:

Peptone	10 Gm.
Dipotassium phosphate	2 Gm.
Agar	15 Gm.
Lactose	1 Gm.
Eosin (yellowish)	0.04 Gm.
Methylene blue	0.01 Gm.
Distilled water	1000 ml.

PREPARATION. The ingredients are mixed and solids dissolved by heating to the boiling point. The solution is made up to volume and dispensed. It is autoclaved for 20 minutes at 15 pounds pressure and stored in the cold. This medium can be purchased in dehydrated form.

Endo's Medium: USE. This medium is primarily employed in the confirmatory test for water analysis, but it is also adaptable to the isolation of non-lactose-fermenting forms.

INGREDIENTS:

Peptone	10 Gm.
Agar	15 Gm.
Basic fuchsin (in alcohol)	0.3 Gm.
Lactose	10 Gm.
Dipotassium phosphate	3.5 Gm.
Sodium sulfite	0.25 Gm.
Distilled water	1000 ml.

PREPARATION. Variation in the strength of dyes and reagents makes it highly advisable to obtain this product in dehydrated form. The dehydrated medium can be purchased from Difco Company. When made in the laboratory, the ingredients other than the dye

and sulfite are combined, dissolved, and autoclaved. The dye is made as a 3 per cent solution and added at the time of use, along with fresh sodium sulfite.

MacConkey's Medium: Use. This is probably the best medium for the differentiation of enteric pathogens from the coli-aerogenes group. Inclusion of crystal violet results in suppression of the Gram-positive organisms while the neutral red colors those colonies splitting lactose and not suppressed by the bile salts.

Ingredients:

Peptone	20	Gm.
Lactose	10	Gm.
Sodium chloride	5	Gm.
Bile salts	5	Gm.
Agar	15	Gm.
Neutral red (1% aqueous)	5	ml.
Distilled water	1000	ml.
(Crystal violet)	0.001	Gm.

Preparation. The agar is dissolved in 500 ml. water by autoclaving or boiling. All other ingredients, except dyes, are dissolved in the remainder of the water by heating in a water bath. The two mixtures are combined and the pH adjusted to 7.4. The resulting agar base is dispensed and stored in the cold after autoclaving at 15 pounds for 20 minutes. At time of use, it is melted; neutral red is added and pour plates are made. The crystal violet can be added, if desired, to the agar base before autoclaving. A slightly different but equally satisfactory dehydrated form can be purchased from Difco Laboratories.

SS Agar (Bile-salts Citrate Agar): Use. This agar is used for the isolation of Salmonella and Shigella from stools. It is prepared to give maximum inhibition of the coli-aerogenes group. It is the solid medium of choice when the only object is detection of these forms since large inocula can be used without jeopardizing the formation of individual colonies.

Ingredients and Preparation. This agar is extremely difficult to prepare in the laboratory. It should be purchased in dehydrated form from Difco Laboratories if possible.

1. Agar base:

Water	1	kg.
Beef extract	5	Gm.
Proteose peptone (Difco)	5	Gm.
Bile salts No. 3 (Difco)	8.5	Gm.
Agar	17	Gm.

The agar is dissolved in half the water by autoclaving. The extract, peptone, and bile salts are dissolved in the remainder of the water. The two solutions are combined and the resulting mixture made up to weight. It is dispensed in bottles or flasks, autoclaved 30 minutes, and stored.

2. The complete medium:

Agar base	1	liter
Lactose (Bacto)	10	Gm.
Sodium citrate ($Na_3C_6H_5O_7.2H_2O$)	8.5	Gm.
Sodium thiosulfate ($Na_2S_2O_3.5H_2O$) (Mallinckrodt)	8.5	Gm.
Ferric citrate pearls U.S.P. VIII, 10% solution	10	ml.
Neutral red (Coleman & Bell), 1% solution	2.5	ml.
Brilliant green (Coleman & Bell), 0.1% solution	0.33	ml.

As required, a suitable amount of agar base is melted; in the proportions given the lactose, sodium citrate, sodium thiosulfate, and ferric citrate solution are added and mixed thoroughly. The pH is adjusted to 7.0. The neutral-red and brilliant-green solutions are then added and the medium dispensed in 15- to 20-ml. amounts in porous-top Petri plates.

Bismuth Sulfite Agar: USE. This agar is employed extensively for the isolation of the salmonellas and especially of the typhoid organism since it is adapted to the use of large amounts of inoculum. It is best used as a matrix for pour plates and on these, members of the salmonella (including *S. typhi*) develop luxuriantly as black, metallic colonies. Most other forms are inhibited.

INGREDIENTS AND PREPARATION:

1. Agar base:

Agar, granulated or powdered	20 Gm.
Beef extract	5 Gm.
Peptone	10 Gm.
Water, hot, to make	1 kg.

The ingredients are dissolved by autoclaving for 15 minutes. If not used at once this base is stored in a refrigerator.

2. Bismuth sulfite mixture:

Bismuth ammonium citrate scales*	6 Gm.
Sodium sulfite, anhydrous†	10–20 Gm.
Dextrose	10 Gm.
Sodium phosphate, anhydrous (Na_2HPO_4)	10 Gm.
Water	200 ml.

The bismuth ammonium citrate scales are dissolved in 50 ml. boiling water, the sodium sulfite in 100 ml. boiling water, and the dextrose in 50 ml. boiling water. The solution of bismuth ammonium citrate and that of sodium sulfite are mixed together and boiled. While boiling the sodium phosphate is admixed. The mixture is allowed to cool; then the dextrose solution is admixed. Water is added to make up the lost weight. This bismuth sulfite mixture is stored in a well-stoppered Pyrex vessel in a dark cupboard at room temperature.

3. Iron citrate-brilliant green solution:

Iron citrate (ferric citrate)	1 Gm.
Water	100 ml.
Brilliant green, 1% solution	12.5 ml.

The iron citrate is dissolved in the water by heating and the brilliant-green solution added. The resulting solution is stored in a well-stoppered Pyrex vessel in a dark cupboard at room temperature.

4. Combination of preparations:

To 1000 ml. hot melted agar base the remaining preparations, in the amounts designated below, are added and mixed thoroughly.

Bismuth sulfite mixture	200 ml.
Iron citrate-brilliant green solution	45 ml.

*The U.S.P. IX "Bismuth and Ammonium Citrate" sold in the United States under the name "Bismuth Ammonium Citrate" may be used.

†The proportion of sulfite may be varied within the given limits as a means of standardizing the medium with different lots of ingredients, e.g., peptone or agar.

The mixture is poured immediately into porous-top Petri dishes, 15 to 20 ml. to each dish. The plates are kept at room temperature for one to two hours and then stored in a refrigerator until required. It is advisable to use these plates within four days after preparation.

Bismuth sulfite agar is not easily adapted to preparation in the laboratory but should be purchased in dehydrated form from Difco Company if possible. The most convenient method of use is to prepare sterile test tubes containing 0.78 Gm. of the powdered medium. When a specimen is to be plated, about 1 Gm. of stool is emulsified in saline, the particles allowed to settle to the bottom, and 1 ml. of the supernatant fluid is pipetted to a sterile Petri dish. To the tube of dry bismuth sulfite medium are added 12 ml. sterile water. This is placed in a boiling water bath until solution is effected. The solution is allowed to cool to around 50° C. and then poured into the Petri plate which is rotated to allow mixing. It is then cooled until solid and is incubated.

Russell's Double-sugar Slants: Use. These slants are used for the separation of the coliform organisms on the basis of fermentation of lactose and dextrose, and for the production of gas from these carbohydrates.

Ingredients:

Beef extract	3 Gm.
Peptone	10 Gm.
Sodium chloride	5 Gm.
Dextrose	1 Gm.
Lactose	10 Gm.
Agar	15 Gm.
Phenol red (0.02% aqueous)	50 ml.
Distilled water	1000 ml.

Preparation. The ingredients are mixed and the solids dissolved by boiling. The volume is restored and the pH adjusted to 7.4. The solution is filtered through cotton if necessary and dispensed in 10-ml. amounts into test tubes. It is autoclaved at 15 pounds for 15 minutes and slanted so as to obtain a deep agar butt and short surface.

Russell's double-sugar slants can also be prepared by melting extract agar and adding lactose and phenol red; in this case only eight minutes of autoclaving will be necessary. Dehydrated Russell's medium can be obtained commercially (Difco). Slants are inoculated with a needle by streaking part of a pure culture over the surface and then stabbing a portion well into the butt of the tube. The reactions to be expected are as follows:

Organisms fermenting both lactose and dextrose with the formation of gas: Escherichia and Aerobacter	Slant yellow; butt yellow and with bubbles
Organisms fermenting dextrose with gas but not affecting lactose: Salmonella and Proteus; some paracoli	Slant red; butt yellow and with bubbles
Organisms fermenting dextrose without formation of gas: Shigella and *S. typhi*. Pseudomonas	Slant red; butt yellow, no gas. Pseudomonas may discolor medium
Organisms fermenting neither dextrose nor lactose; Alkaligenes	Slant red; butt yellow

Kligler's Iron Agar: Use. This agar has the same use as Russell's slants, except that, through the inclusion of an iron salt (ferrous ammonium sulfate, 0.2 Gm. per liter) and sodium thiosulfate (0.08 Gm. per liter), organisms capable of producing hydrogen sulfide give a black discoloration, thus combining the properties of Russell's medium with a test for hydrogen-sulfide production.

INGREDIENTS AND PREPARATION. Since this medium is not especially stable when prepared in large amounts, it is more economical to purchase it in the dehydrated form. The reactions are comparable to those described for Russell's slant except that most salmonellas and some proteus members produce a black discoloration.

Krumweide's triple sugar agar is like Russell's except that sucrose is also added in an amount equal to the lactose.

Triple sugar iron (*T.S.I.*) (Hajna, 1945) is a more complicated and very satisfactory medium for the differentiation of the enteric bacteria. It may be purchased from the Baltimore Biological Laboratory.

Tomato-juice Agar: USE. This is used for lactobacilli.

INGREDIENTS:

Solution A:

Peptone (Bacto-Difco)	10 Gm.
Peptonized milk (Bacto-Difco)	10 Gm.
Tomato juice (from canned tomatoes)	400 ml.

Solution B:

Agar-agar (Bacto-Difco)	25 Gm.
Distilled water	600 ml.

PREPARATION. Solution A is prepared by adding the peptone to the tomato juice and mixing well. Then the peptonized milk is added. After mixing well the preparation is placed in an Arnold Sterilizer and gradually heated to 95° to 100° C.

Solution B is made by adding agar-agar to the distilled water and mixing well. The mixture is heated for 30 minutes in steam (Arnold Sterilizer) before autoclaving for 30 minutes at 15 pounds pressure.

Solutions A and B are mixed while hot. The reaction is determined and the pH adjusted to 6.0 with N/1 sodium hydroxide. The mixture is heated for 10 minutes in steam (Arnold Sterilizer). The reaction is determined and, if necessary, the pH is adjusted to 6.0 using N/1 sodium hydroxide. The reaction is then readjusted to pH 5.2 using lactic acid. After heating the mixture for 10 minutes in steam (Arnold Sterilizer), the reaction is again determined and, if necessary, corrected to pH 5.2. The solution is now filtered through a fine layer of nonabsorbent cotton and dispensed into desired containers. It is sterilized for eight minutes at 15 pounds pressure (autoclave 121.6° C.).

Aronson's Medium: USE. Aronson's medium is used for the isolation of *V. comma*.

INGREDIENTS:

Agar	30 Gm.
Beef extract	3 Gm.
Peptone	10 Gm.
Sodium chloride	5 Gm.
Distilled water	1000 ml.

The above ingredients serve as a base and can be combined and stored exactly as described for extract agar. The following solutions are prepared separately and are sufficient for 100 ml. of agar base:

Sodium carbonate (anhydrous), 10%	6 ml.
Saccharose 20%	5 ml.
Dextrin 20%	5 ml.
Sodium sulfite 10%	2 ml.
Basic fuchsin (saturated alcoholic sol.)	0.4 ml.

PREPARATION. To the melted agar base is added the sodium carbonate. This mixture is heated over steam for 15 minutes. The remaining solutions are sterilized without pres-

sure for 30 minutes and then added to the base. Any precipitate is allowed to settle out before pour plates are made.

Cholera organisms grow rapidly on this medium and take the color of the fuchsin. Owing to the alkalinity of the matrix, most other forms do not grow or grow more poorly than *V. comma.*

Dieudonne's Medium: Use. This is used for the isolation of *V. comma.*

Ingredients and Preparation. To 70 ml. of the hot melted base, described above, 30 ml. of equal parts defibrinated blood and normal sodium hydroxide solution are added. Pour plates are prepared and allowed to dry with the covers partially removed, either in the incubator for overnight or in an oven at 60° C. for a few minutes. Cholera vibrios and others of the group grow well on this alkaline surface while most other fecal organisms do not.

Alkaline Peptone Water: Use. Alkaline peptone water is used for the cholera red test (nitro-indol reaction).

Ingredients. The ingredients are the same as for peptone water (p. 352).

Preparation. The ingredients are mixed and dissolved with heat. The pH is adjusted to 8.0 to 8.4 before the preparation is tubed in 10-ml. amounts and autoclaved.

The cholera red test is performed by inoculating tubes and allowing growth to take place for 24 hours. At the end of this period, several drops of concentrated sulfuric acid are added and the mixture observed for the development of a pink coloration. Nitro-indol is produced irregularly by different peptones and by different lots of the same peptone. A positive control should be run with each new lot.

Verwoort's Medium: Use. This medium is used for the cultivation of leptospira.

Ingredients and Preparation. To 1.5 liters tap water, 1.5 Gm. Bacto-peptone are added. After boiling, 6 ml. phosphate solution, prepared by dissolving 0.35 Gm. monobasic potassium phosphate (KH_2PO_4) and 1.33 Gm. dibasic sodium phosphate (Na_2HPO_4) in 100 ml. distilled water, are added. When this mixture is brought to the boiling point, 300 ml. Ringer's solution (0.8 per cent NaCl, 0.02 per cent $CaCl_2$, 0.02 per cent KCl, 0.02 per cent $NaHCO_3$) are added. Boiling is continued and 150 ml. Sørenson's phosphate buffer of pH 7.4 (120.6 ml. M/15 Na_2HPO_4 plus 29.4 ml. M/15 KH_2PO_4) are added. Boiling is continued until precipitation is complete (about 30 minutes). The mixture is cooled in the refrigerator overnight and then filtered. The pH is checked; it should be between 6.8 and 7.2. The medium is bottled and autoclaved at 15 pounds for 15 minutes. Before use, 12 per cent sterile rabbit serum (the serum should be obtained from a rabbit deprived of food for 24 hours) and 8 ml. citrated rabbit blood, which has been laked by the addition of 70 ml. water, are added. The mixture is tubed in 2.5- to 3-ml. amounts and inactivated for 30 minutes at 56° C. It is incubated to determine sterility.

When this medium cannot be prepared, successful results can often be obtained by adding serum and laked blood to Ringer's solution or other mildly alkaline matrix.

Sabouraud's Medium: Use. This is utilized in the cultivation and maintenance of fungi.

Ingredients:

Maltose (technical)	40 Gm.
Neopeptone	10 Gm.
Agar	20 Gm.
Distilled water	1000 ml.

Preparation. The ingredients are mixed and the solids are dissolved by heating. The mixture is filtered through paper, dispensed in 20-ml. amounts in large test tubes, autoclaved for 10 minutes at 15 pounds, slanted, and allowed to solidify. The final reaction should be well on the acid side (pH 5.6).

This medium, without maltose, is used to keep stock cultures. Growth is so slow that frequent transplants are not necessary.

Cornmeal Agar: Use. Cornmeal agar is used primarily for the cultivation of yeasts but may also be of use for encouraging the development of spore-forming stages by fungi.

Ingredients:

Yellow cornmeal	40 Gm.
Agar	15 Gm.
Water	1000 ml.

Preparation. The cornmeal is steeped in 500 ml. water at 65° C. for one hour and then filtered through paper. The agar is dissolved in the remainder of the water by heat. The agar solution and cornmeal extract are mixed and filtered through cotton while hot. The preparation is then tubed, autoclaved at 15 pounds pressure for 15 minutes, and slanted.

Nutrient Broth for Water Analysis: Use. This is a standard broth from which the official media for water analysis are prepared.

Ingredients:

Beef extract	3 Gm.
Peptone	5 Gm.
Distilled water	1000 ml.

Preparation. The ingredients are mixed and the solids are dissolved by boiling. The solution is made up to volume, dispensed, and autoclaved at 15 pounds for 15 minutes.

To prepare lactose broth, the reaction of nutrient broth is checked and the pH is adjusted to 6.6 to 6.9. After adding 0.5 per cent lactose, the broth is dispensed in fermentation tubes and autoclaved at 15 pounds for 15 minutes.

Nutrient agar is prepared by adding 15 Gm. of agar per liter of broth, clarifying if necessary, and autoclaving as above.

Tryptose-glucose-extract-milk Agar: Use. This is the standard medium for plate counts in milk analysis.

Ingredients:

Beef extract	3 Gm.
Tryptone	5 Gm.
Glucose	1 Gm.
Agar	15 Gm.
Water	1000 ml.
(Skim milk)	10 ml.

Preparation. The ingredients, except milk, are mixed and heated until the solids are dissolved. The solution is made up to volume. The milk is added and the mixture heated to boiling. It is clarified, if necessary, and autoclaved at 20 pounds for 15 minutes. The milk can be omitted when dilutions of 1 : 10 or less are to be used. Powdered milk, reconstructed to desired strength, can be substituted if more convenient.

Crystal-violet Lactose Broth: Use. This is an accepted liquid confirmatory medium in water analysis.

Ingredients:

Peptone	5 Gm.
Lactose	5 Gm.
Dipotassium phosphate	5 Gm.
Monopotassium phosphate	1 Gm.
Crystal violet (0.0143 per cent solution)	10 ml.
Distilled water	1000 ml.

Preparation. The solid ingredients are dissolved in the water by heating gently. The solution is cooled and pH adjusted to 7.3 to 7.5. The dye solution is added. The broth is brought up to volume, dispensed in fermentation tubes, and autoclaved.

Fuchsin Lactose Broth: Use. This is an accepted medium for the confirmatory test of water.

Ingredients:

Beef extract	3 Gm.
Peptone	5 Gm.
Lactose	5 Gm.
Basic fuchsin (0.1% solution)	15 ml.
Distilled water	1000 ml.

Preparation. All the ingredients except the dye are mixed and heated until the solids are dissolved. The solution is cooled and the pH adjusted to 6.6 to 7.0. The dye solution is added. The broth is brought up to volume, tubed, and autoclaved.

Formate-ricinoleate broth and brilliant green-bile broth are satisfactory confirmatory media but more difficult to prepare from basic ingredients. Both, as well as fuchsin lactose broth and crystal-violet lactose broth, can be obtained in dehydrated form from the Difco Laboratories. It must be remembered that with any of the confirmatory media it is necessary to provide twice as much medium as water sample. When large amounts of water are cultured, a more concentrated basic medium must be used in order to meet this regulation. Ten ml. of the standard media described above are satisfactory for 5-ml. samples or less.

Violet-red Bile Agar: Use. This is an official medium in the presumptive test for coliforms by the pour-plate method.

Ingredients. It is recommended by Standard Methods that, in the interest of uniformity, this medium be purchased in dehydrated form from the Difco Laboratories. The composition, as given by that laboratory, is as follows:

Peptone	10	Gm.
Lactose	10	Gm.
Bile salts	1	Gm.
Yeast extract	5	Gm.
Agar	15	Gm.
Neutral red	0.05	Gm.
Crystal violet	0.004	Gm.

A satisfactory alternative for this medium is "Desoxycholate Agar," prepared by the Baltimore Biological Laboratory.

Reagents

Physiological Saline Solution. This solution is prepared by dissolving 8.5 Gm. sodium chloride in 1 liter distilled water. This is dispensed in suitable containers and autoclaved.

Buffered Glycerol: Use. Buffered glycerol is used for the preservation of virus-containing tissues.

Ingredients:

Citric acid (2.1 Gm.% in distilled water)	9.15 ml.
Disodium phosphate, anhydrous (2.48 Gm.% in distilled water)	90.85 ml.
Glycerin, neutral (C.P.)	100.00 ml.

Preparation. The first two ingredients are mixed to form the buffer which should have a pH of 7.4. Then glycerin is added and the preparation dispensed in specimen bottles and autoclaved at 15 pounds pressure for 30 minutes.

Dichromate Cleaning Solution. With the aid of heat, 300 Gm. potassium dichromate (technical) are dissolved in 2 liters water. To this are added cautiously 1850 ml. commercial concentrated sulfuric acid. *Caution: Never pour the aqueous solution into the acid. Handle with care. Avoid contact with flesh and clothing.*

Citrate Bottles for Blood Cultures. Citrate bottles are prepared by adding 0.5 ml. of 10 per cent sodium citrate to a bottle and sterilizing by autoclaving.

Sample Bottles for Chlorinated Waters. Sodium thiosulfate solution is prepared by dissolving 1.5 Gm. sodium thiosulfate in 100 ml. distilled water. One-half ml. of this solution is placed in each clean bottle. (This amount has been found sufficient to reduce completely residual chlorine in an amount up to two parts per million in a sample of 130 ml. water.) The bottles with the solution are sterilized for 15 minutes at 20 pounds pressure.

CHAPTER 12

Staining Methods and Special Procedures

The morphology of the different bacteria is studied chiefly by means of stained smears, and the appearance of certain species of bacteria stained by special methods may be sufficiently characteristic to identify them. It is advisable to use clean new slides. New slides may be cleaned adequately by immersing them in 95 per cent alcohol and rubbing them dry. If old slides are used they should be passed through acid cleaning solution.

Preparation of Films. For examination of cultures from a solid medium, a small loopful of distilled water or saline is placed on a clean glass slide (or coverslip, cleaned as directed on p. 387), a colony is touched with the tip of a platinum needle, and a minute amount of the growth is stirred into the loopful of water so as to give a faint turbidity. The mistake of adding too much bacterial growth is very frequently made. The drop is spread into a thin film and allowed to dry in air. With fluid medium, a drop is spread on the slide without dilution and allowed to dry in air.

The smear is fixed by passing the coverslip, film side up, quickly through a flame three times. On a slide it may be fixed by passing the slide through a flame four or five times. Or the films may be flooded with absolute ethyl or methyl alcohol, or glacial acetic acid, and after one or two minutes washed with water and dried in air. Films to be examined for tubercle bacilli should be fixed a second time in alcohol after heat fixation, but as a rule, heat fixation is adequate. The film should be allowed to cool before staining.

For routine work it is recommended that films be stained with Loeffler's methylene blue and by Gram's method. Ziehl-Neelsen's carbolfuchsin, freshly diluted with water (1 : 10), may be substituted for methylene blue.

It is convenient to keep on hand saturated alcoholic solutions of the various dyes from which to prepare the ordinary bacterial stains. The list shown in Table 42 indicates the quantities of the different dyes required to obtain saturated solutions.

Liquid petrolatum is by far the best mounting medium. It is always of proper consistency and is never sticky. Unlike Canada balsam, it does not develop any acidity and permanent mounts retain their color for at least two years. This is particularly important with preparations of blood, malarial parasites, trypanosomes, intestinal flagellates, etc., in which a well-stained chromatin is necessary for identification. It is also useful for mounting small insects and sporangia of molds. Furthermore, it has superior optical properties. For permanent preparations the border of the coverglass should be sealed with gold size or some other cement.

Hanging-drop Preparations. To prepare a hanging drop a loopful of the young (4- to 8-hour) broth culture (or a loopful of salt solution into which is then emulsified a *small* amount of growth from an agar slant) is placed in the center of a thin coverglass. Small drops of oil are placed on opposite sides of the concavity of a hollow-ground slide; the coverslip is inverted over the concavity so that the drop of bacterial suspension hangs from the coverslip without touching the slide. The drop must not touch the oil. If a hol-

Table 42

SOLUBILITY OF DYES IN WATER AND ALCOHOL

Name of Dye	*Per Cent Soluble in:*	
	Water	*95 Per Cent Alcohol*
Bismarck brown Y	1.36	1.08
Crystal violet (chloride)	1.68	13.87
Eosin Y (Na salt)	44.20	2.18
Erythrosin (Na salt)	11.10	1.87
Fuchsin (basic) rosaniline (chloride)	0.39	8.16
Para-rosaniline (chloride)	0.26	5.93
Para-rosaniline (acetate)	4.15	13.63
New fuchsin (chloride)	1.13	3.20
Janus green	5.18	1.12
Malachite green (oxalate)	7.60	7.52
Methyl violet	2.93	15.21
Methylene blue (chloride)	3.55	1.48
Neutral red (chloride)	5.64	2.45
Picric acid	1.18	8.96
Pyronin B (iodide)	0.07	1.08
Pyronin G	8.96	0.60
Safranin	5.45	3.41
Thionin	0.25	0.25
Toluidine blue (O)	3.82	0.57

low-ground slide is not available, the coverslip may be ringed thickly with petroleum jelly, and inverted over an ordinary slide.

In the microscopic examination the concave mirror should be used. The light coming through the diaphragm of the microscope is reduced. Using the low-power objective the edge of the drop is located. Then the high-power (dry) objective is moved into place, and the light readjusted. After focusing on the edge of the drop the slide is moved about and the bacteria observed for motility. The oil-immersion objective can be used if a drop of thin oil is placed on the coverslip, but this is not advisable as a routine procedure.

Hanging-drop preparations must be sterilized before cleaning. During examination care must be taken not to break the coverslip because of the danger of contaminating the lens and stage of the microscope.

Stains for General Use

The following stains are most commonly used in the bacteriologic laboratory:

Loeffler's Alkaline Methylene Blue. The following ingredients are used:

Methylene blue (certified)	0.3 Gm.
Ethyl alcohol	30 ml.
Potassium hydroxide (0.01% aqueous)	100 ml.

PREPARATION. The dye is dissolved in the alcohol and this solution is mixed with the hydroxide solution. The mixture is filtered.

USE. The fixed smear is flooded with the stain. After one minute it is washed with water. The bacteria and most cellular elements stain blue. To demonstrate *Borrelia vincentii* the smear is stained 10 minutes or more.

Gram's Method. This is the most important staining method in bacteriologic technic. It is, however, likely to give unsatisfactory results in the hands of the inexperienced and in using it the following points must be kept in mind.

Laboratory cultures (subcultures) which have been carried over for years frequently lose their Gram characteristics. Cultures which are several days old or dead or degenerated do not stain characteristically. The iodine solution deteriorates and becomes light in color; it should be of a rich port-wine shade. Decolorization, best observed over a white background, should be stopped as soon as no more violet stain is seen to stream out from the preparation. Decolorization can be controlled by making a preparation of a known Gram-positive and a known Gram-negative organism on either side of the smear to be stained. The preparation should be thin and evenly spread.

INGREDIENTS. The following are used in making Gram's stain (Lillie's modification):

Solution A:	
Crystal violet	2 Gm.
Ethyl alcohol (95%)	20 ml.
Ammonium oxalate (1%) in distilled water	80 ml.
Solution B:	
Potassium iodide	2 Gm.
Crystal iodine	1 Gm.
Distilled water	300 ml.
Solution C:	
Acetone	
Solution D:	
Safranin	1 Gm.
Distilled water	100 ml.

Solutions A, B, and D are prepared by mixing their respective ingredients and filtering. Solution A keeps well.

USE. The fixed smear is covered with solution A for 30 seconds. It is then rinsed, flooded with solution B for 30 seconds, rinsed, and decolorized with acetone (solution C). (Hucker uses 70 ml. of 95 per cent ethyl alcohol plus 30 ml. acetone for decolorization.) Following decolorization the smear is flooded with solution D for 10 seconds.

REACTIONS. In the following tabulation are listed the Gram-staining reactions of important bacteria:

GRAM-NEGATIVE	GRAM-POSITIVE
Cocci	*Cocci*
Meningococcus	Staphylococcus group
N. catarrhalis	Streptococcus group
Gonococcus	Pneumococcus group
	G. tetragena
Bacilli	*Bacilli*
K. pneumoniae	*Corynebacterium* group
P. aeruginosa	*C. tetani*
F. dentium	*M. tuberculosis*
E. coli group	*M. leprae*
Salmonella group	*B. anthracis*
Shigella group	*C. welchii* and other spore-forming anaerobes
M. mallei	
C. chauvoeii	
P. tularensis	
P. pestis	
H. influenzae	

GRAM-NEGATIVE		GRAM-POSITIVE	
Bacilli		*Spirilla*	
H. pertussis		None important	
H. ducreyi			
H. lacunatus			
H. conjunctivitidis			
Brucella group			
Spirilla		*Gram amphophil (variable)*	
Cholera and allied forms		Molds	
Mouth spirals		Yeasts	
		Protozoa	

Methods for Staining Acid-fast Bacilli: ZIEHL-NEELSEN'S METHOD. Ziehl-Neelsen's stain utilizes the following ingredients:

Solution A:

Basic fuchsin	1 Gm.
Ethyl alcohol	10 ml.
Phenol (5% aqueous solution)	90 ml.

Solution B:

Hydrochloric acid (concentrated)	3 ml.
Ethyl alcohol	97 ml.

Solution C:

Loeffler's alkaline methylene blue

USE. The fixed smear is covered with carbolfuchsin and steamed gently over a flame for three minutes. The stain is renewed repeatedly to prevent drying on the slide. The carbolfuchsin may be allowed to act, without steaming, for 30 minutes or overnight at room temperature. Five minutes suffice for the leprosy bacilli. After staining with carbolfuchsin, the smear is washed with water, decolorized with acid alcohol until thin areas are colorless, and again washed with water. It is counterstained with Loeffler's alkaline methylene blue for 30 to 60 seconds, washed with water, and blotted dry. To avoid overstaining, some prefer to use a 1 : 10 dilution of Loeffler's stain, just long enough to give the background a faint-blue color.

In place of solutions B and C, Hanks advises a 1 : 4 dilution of Gabbett's solution (2 Gm. methylene blue, 100 ml. of 25 per cent sulfuric acid). After staining with solution A, the slides are washed and immersed in diluted Gabbett's solution for five minutes (or longer if the film shows any residual redness). They are then washed in water and dried. In this method there are fewer manipulations, and loss of bacilli from the films is minimized.

Stained by Ziehl-Neelsen's method, acid-fast organisms appear as red rods against a blue background.

Leprosy bacilli often are decolorized more readily than tubercle bacilli, and if leprosy is suspected, decolorization with acid alcohol should be light. Some prefer to use a 5 to 20 per cent aqueous solution of sulfuric acid, as in the diluted Gabbett's solution.

Smegma bacilli also are usually more easily decolorized than tubercle bacilli, but special staining methods to differentiate them on this basis are not entirely reliable. Pappenheim's method is recommended.

PAPPENHEIM'S METHOD OF DIFFERENTIATION. This is the standard method. After steaming the smear in carbolfuchsin, the stain is poured off and, without washing, the film is treated with the following: Corallin, 1 Gm.; absolute alcohol, 100 ml.; methylene blue, 0.66 Gm., and glycerin, 20 ml. The film is flooded several times with the mixture, which

is allowed to drain off slowly following each application. It is then washed in water and mounted. The smegma bacillus is decolorized by the corallin and alcohol but the tubercle bacilli remain red.

Fontes' Method. Fontes has devised a method of staining acid-fast bacilli which will also stain those elements which have lost their acid-fast properties. The so-called Much granules are also characteristically stained. The method is to stain the preparation with carbolfuchsin, decolorize with acid alcohol, then carry through the various steps of the Gram method, counterstaining, however, with Bismarck brown. By this method the acid-fast tubercle bacilli show as red rods dotted with violet granules. Those which do not fully retain acid-fast properties show as zigzag violet lines.

Beck's Stain. This is similar to Neisser's stain, but crystal violet is used instead of methylene blue. This is used for the demonstration of diphtheria bacilli. Two solutions are prepared.

Solution A:

Crystal violet (saturated alcoholic sol.)	10	ml.
Acetic acid (4.3% aqueous solution)	90	ml.

Solution B:

Bismarck brown	0.48	Gm.
Distilled water	125	ml.

To prepare the Bismarck brown solution the water is heated to boiling. The stain is added and boiling continued for two minutes. The solution is filtered and cooled.

Use. Solution A is applied for 2 minutes. The slide is washed in water and excess moisture drained off. It is then stained with solution B for 30 seconds, washed and dried. The metachromatic granules of corynebacteria stain purple while the remainder of the cell body and most other bacteria take the brown color. This stain has been highly satisfactory for demonstrating the diphtheria bacillus. When it is not available, Loeffler's alkaline methylene blue can be substituted.

Wayson's Stain. This stain, originally designed for the study of plague, has attained a wide general use. It has been especially valuable in staining *Pasteurella tularensis* and *Streptobacillus moniliformis*. It is a good stain for demonstrating capsules.

Ingredients:

Fuchsin	0.2	Gm.
Methylene blue	0.75	Gm.
Phenol (99.5%)	10.0	ml.
Alcohol (absolute ethyl)	20.0	ml.
Distilled water	200.0	ml.

The dyes are dissolved in separate portions of the alcohol; the phenol is dissolved in the water. The dissolved dyes are added to the phenolized water and the solution filtered.

Use. This stain is applied undiluted for 10 to 30 seconds. It gives a clear pink-and-blue differentiation.

Hiss' Capsule Stain. The following ingredients are used in the preparation of this stain:

Solution A:

(Use the crystal violet solution described for the Gram stain.)

Solution B:

Copper sulfate	20	Gm.
Distilled water	100	ml.

Use. The organisms under study are mixed with a drop of serum on a slide, dried, and fixed gently with heat. The slide is stained with solution A, heated for a few seconds

over a flame, and excess stain is washed off with solution B. The excess copper sulfate is blotted off and the slide dried. Bacteria will be stained purple and their capsules will be a light blue.

Dorner's Spore Stain. This is prepared as follows:

Solution A:

Nigrosin	10 Gm.
Distilled water	100 ml.
Formalin	0.5 ml.

Solution B:

Freshly filtered carbolfuchsin as described for the Ziehl-Neelsen stain.

To make solution A, the nigrosin is added to boiling water and boiling continued for 30 minutes. After cooling, the formalin, which serves merely as a preservative, is added. The solution is filtered twice through paper and stored in 5-ml. amounts in test tubes.

Use. A heavy suspension of the organism under study is made in 2 to 3 drops of distilled water in a small test tube. An equal quantity of carbolfuchsin is added. The mixture is allowed to stand in a boiling water bath 10 to 12 minutes. On a slide, one loopful of the stained preparation is mixed with one loopful of a saturated aqueous solution of nigrosin. This is smeared as thinly as possible and dried rapidly. The spores are stained red, the bodies of the bacteria are almost colorless and stand out against the dark-gray background of nigrosin.

Polychrome (Romanowsky) Stains. Wright's stain, Giemsa's stain, and the May-Grünwald-Giemsa stain are described in the section on hematology. We recommend the purchase of certified stains from reliable dealers. These polychrome stains are also used to demonstrate malarial parasites, trypanosomes and other protozoa, spirochetes, rickettsiae, and the intracellular inclusion bodies of certain filtrable virus infections.

Fontana-Tribondeau's Spirochete Stain. The following are prepared:

Solution A:

Acetic acid (glacial)	1 ml.
40% formalin	10 ml.
Distilled water	100 ml.

Solution B:

Tannic acid	5 Gm.
1% phenol	100 ml.

Solution C:

5% silver nitrate solution	50 ml.

A few milliliters of the silver nitrate solution are reserved. To the remainder is added, drop by drop, concentrated ammonia solution until the sepia precipitate which forms redissolves. The silver nitrate solution is shaken and stirred constantly during the addition of the ammonia. Then some of the reserved silver nitrate solution is added, drop by drop, until there occurs a slight clouding, which persists on shaking. This solution will remain useful for several months. Occasionally it should be poured into a clean receptacle, and if it has become clear, a few more drops of 5 per cent silver nitrate are added.

Use. On a clean slide, a thick film of fluid from a chancre, or other material containing spirochetes, is made and allowed to dry in the air. The film is covered with solution A for one minute. It is washed thoroughly with distilled water, covered with solution B, and heated until the fluid steams. It is again washed with distilled water. It is now covered with solution C and heated until the fluid steams. This is allowed to act for 30 seconds.

The slide is washed with water and dried in air or blotted. The stained film should be a maroon color and the spirochetes stained dark brown or black.

India-ink relief method of demonstrating spirochetes:

A loopful of material to be examined is mixed on a slide with a drop of India-ink, spread smoothly, and dried.

The microörganisms appear unstained on a dark background.

Castaneda's Stain for Rickettsiae. The following ingredients are prepared:

Solution A:	
Potassium dihydrogen phosphate	1 Gm.
Disodium phosphate ($Na_2HPO_4.12H_2O$)	25 Gm.
Distilled water	200 ml.
Formalin	1 ml.

Each salt is dissolved in 100 ml. of water, the two solutions are mixed together, and the formalin added as a preservative. The pH of this buffer solution should be 7.5.

Solution B:	
Methyl alcohol	100 ml.
Methyl blue	1 Gm.
Solution C:	
Safranin "O" (0.2%)	25 ml.
Acetic acid (0.1%)	75 ml.

Use. To 20 ml. buffer, 1 ml. formalin and 0.15 ml. solution B are added. The slide is stained with this mixture for three minutes and, at the end of this time, the stain is poured off but the slide is not washed. The smear is covered with the safranin solution for two to five seconds and washed immediately in running water.

Cotton-blue Lactophenol. This preparation is used for staining fungi.

Phenol crystals	20 Gm.
Lactic acid	20 ml.
Glycerol	40 ml.
Distilled water	20 ml.
Cotton blue	0.05 Gm.

The phenol is dissolved in the liquids by heating gently. The dye is added when solution is complete.

Use. Specimens are cleared in 10 per cent potassium hydroxide and washed gently in water. The washed specimens are placed in a drop of the stain and covered with a coverslip. The fungus elements will be dark blue, the remainder of the structures a light blue. Permanent mounts can be made by using glycerin as a clearing agent rather than hydroxide, and by sealing the coverslip to the slide.

Miscellaneous Procedures

Aspiration of Material from Chancres. The adapter end of a 5- to 10-ml. syringe barrel is cut off so as to form a cylinder. Then the plunger is inserted in the reverse direction so that the smooth end (uncut end) of the barrel and the smooth end of the plunger are together. The chancre is cleansed and any exudates or scabs are removed. The smooth end of the syringe barrel is placed over the lesion and gentle suction is exerted by withdrawing the plunger. A drop or two of lymph will exude and this can be picked up on the slide for darkfield examination. This method is especially useful on lesions located in areas other than the penis.

Delayed Planting of Gonococcus Cultures. A 1 per cent aqueous solution of gentian violet is added to distilled water to make a final dilution of 1 : 15,000 (0.1 ml. of 1

per cent solution of gentian violet in 14.9 ml. distilled water). This solution is autoclaved at 10 pounds for 10 minutes. An equal amount of sterile defibrinated horse blood is added to the dye to make the final solution of 1 : 30,000 gentian violet (15 ml. defibrinated horse blood to 15 ml. of 1 : 15,000 gentian violet). Test tubes 6 × 50 mm. are used with cork stoppers to fit (No. 000 xxx). The tubes which are to contain the blood-dye solution are autoclaved at 15 pounds for 20 minutes. A 10-ml. sterile pipet is used to fill the tubes approximately two-thirds full with the blood-dye solution. The tubes are plugged with cork stoppers which have been previously dipped in melted paraffin and kept under 70 per cent alcohol. The tubes are inverted and the stoppers sealed with hot paraffin. A similar number of tubes is used for keeping swabs sterile. The cork stoppers are dipped in melted paraffin. Round toothpicks are cut in half, swabs are made at the cut ends of the toothpicks, and the pointed ends are inserted into the stoppers. The swabs attached to stoppers are placed in test tubes and autoclaved at 15 pounds for 20 minutes. The tubes are inverted and the stoppers sealed with hot paraffin.

In collecting the specimen, the cork to which the swab is attached is removed from the tube. The swab is brought in contact with the exudate, then placed in a tube containing the blood-dye. The tube is closed with cork to which an applicator is attached. When obtaining material from the cervix,* dressing forceps are used for grasping the cork and the swab.

Demonstration of Tubercle Bacilli by Guinea-pig Inoculation. Pigs weighing about 250 Gm. are used and, when possible, these animals are injected in duplicate. Sputum should be treated with 3 per cent hydrochloric acid or 4 per cent sodium hydroxide for two hours in order to kill extraneous organisms. The pH should then be brought back to neutral, the specimen centrifuged, and 1 to 2 ml. of the sedimented portion injected.

Gastric washings rarely need more treatment than neutralization and centrifugation. Urine can be concentrated as described above for direct examination. Pleural fluid and cerebrospinal fluid are best handled by using the guinea pig as a filter, injecting 3 ml. daily into the same pig until 20 or 30 ml. have been given. Injection into the guinea pig should be made in the subcutaneous spaces of the thigh or lower abdomen, taking care that the material is not injected into the peritoneal cavity. An inoculum greater than 3 ml. should not be used. The inoculum must be free from other pathogens and not so acid or alkaline as to prove irritating. The animals are inspected from time to time, especially for any enlargement of the inguinal lymph nodes. At the end of six weeks they can be autopsied. Caseous lesions of lymph nodes, spleen, and liver are especially sought. If any are found, smears are made and stained by the Ziehl-Neelsen method. If acid-fast bacilli are not found in typical caseous lesions, the tissue should be examined histologically by a pathologist and a new guinea pig inoculated with some of the material. If guinea pigs die before the allotted six weeks, sections of the spleen and liver should be examined histologically when possible.

Bile-solubility Test. When fluid bile is available it can be added to broth cultures of organisms suspected to be pneumococci, using 0.2 ml. of the bile per 1 ml. of the broth. The tube of bile-broth mixture is placed in the incubator for 15 minutes and the turbidity compared with that of a tube of untreated broth culture.

Powdered bile can be purchased (Difco) and is quite satisfactory. It can be placed directly on plate colonies and these will disappear if they are composed of pneumococci while the viridans type of streptococcus remains unaffected. Sodium lauryl sulfate, sold by Dupont as "duponol" can be used by applying it directly to the culture plate or it can be added to broth cultures, using 0.1 ml. of a 0.2 per cent solution per 0.6 ml. of broth.

Sodium desoxycholate is also able to lyse pneumococci. Two drops of a 10 per cent

*A small amount of semisolid agar (1 to 2 ml.) in the bottom of the tube, and an ordinary nasopharyngeal swab may be used for this same purpose. Such tubes must be kept in an upright position.

solution will clear 1 ml. of broth. It is wise when using sodium desoxycholate to adjust the pH of the broth to neutral before adding the reagent.

Fibrinolysin Test for Beta Hemolytic Streptococci. Two-tenths ml. oxalated human plasma (0.02 Gm. potassium oxalate to 10 ml. blood) is diluted with 0.8 ml. physiological salt solution. To this is added 0.5 ml. of a young (18- to 25-hour) turbid broth culture of the streptococcus to be tested. Following immediate mixing, 0.25 ml. of a 0.25 per cent aqueous solution of calcium chloride is added. After mixing, this is placed in a water bath at 37° C. In about 10 minutes there should be a solid coagulum. The tube is observed frequently and the time when its contents become completely fluid is noted.

Plasma from individuals who have recovered recently from hemolytic streptococcus infections is not suitable for the test.

Antifibrinolysin. When normal blood is used complete dissolution occurs within an hour. The presence of antifibrinolysin is demonstrated by prolonged lysing time. The time required for complete lysis of the plasma-clot is used as an index of antifibrinolysin concentration as indicated in the following scheme:

0 = complete dissolution in less than 1 hour
1 = " " " 1 to 4 hours
2 = " " " 4 to 8 "
3 = " " " 8 to 24 "
4 = no complete dissolution in 24 hours.

Penicillin*

Methods of Assay of Penicillin (Based Largely on Fleming: "Penicillin," The Blakiston Company, 1946). The *"unit"* of penicillin is a purely arbitrary standard based upon its biologic activity. As originally defined, an Oxford unit was the minimum amount of penicillin which, when dissolved in 50 ml. of meat extract broth, would inhibit the growth of a special (standard) strain of *Staphylococcus aureus*. Since penicillin has been obtained in relatively pure form, the unit has been redefined as equivalent to the activity of 0.0006 mg. of a preparation of the pure crystalline sodium salt of penicillin II (G) maintained as a standard at the National Institute of Medical Research, London. The International Conference on the Standardization of Penicillin recommended that one of two special strains of *S. aureus* be used for titrating penicillin preparations. These are kept in stock in the National Collections of Type Cultures in this country and in London. Other species have been used, however, including the pneumococcus, hemolytic streptococcus, and *B. subtilis*.

Strict asepsis must be observed in all the manipulations described below. Samples of Standard Penicillin may be obtained from the Food and Drug Administration of the U. S. Federal Security Agency, Washington, D.C.

Agar Plate Method. The preparation of agar cups is described on p. 276. The Petri dishes must be flat and even, so that the culture medium will have uniform depth. Serial dilutions of the solution to be tested and also of a penicillin solution of known strength, preferably including dilutions containing 2, 1, 0.5, and 0.25 units per ml. are prepared in broth. Into each of the cups are placed a few drops of one of the dilutions, so as nearly to fill the cups. These are labeled and incubated overnight. Then the diameter of the zone around each cup in which growth has been inhibited is measured. Some of the dilutions of the solution to be tested should correspond in activity to those of the standard solution. The strength (activity) of the former can then be determined by direct comparison and simple calculation.

In place of making cups, one may use small glass or porcelain cylinders 7 mm. in external diameter and 8 to 10 mm. long. These are placed erect on an agar plate and fitted closely so that there is no leakage when fluid is placed in the cylinders (see p.

*This section has been added by the editor.

371). The agar-plate method is a relatively crude procedure, and it is influenced by the diffusibility as well as the potency of the penicillin.

Serial Dilution Method. In test tubes, serial dilutions in broth of the solution to be tested and of a standard solution of penicillin of known strength are made. Inoculate each tube with a loopful of a broth culture of the standard strain of *S. aureus* and incubate overnight. The highest dilution in each series in which growth is inhibited is determined by inspection and the strength of the solution is calculated as above.



Assay plate. Test organism—staphylococcus. The cylinders contained 4, 2, 1, 0.5, and 0.25 units of penicillin per milliliter. (From Fleming: Penicillin, Philadelphia, The Blakiston Company.)

Slide Cell Method (Micromethod) for Assay in Blood Serum. A 10 per cent normal human serum being used as diluent, serial dilutions of the serum to be tested are prepared in 50-cu. mm. quantities on a paraffined glass slide. Each is inoculated with a loopful of such a dilution of a 24-hour broth culture of the standard strain of *S. aureus* (about 1 : 40,000) that the inoculum yields not over 50 colonies. A normal human serum to which has been added a measured amount of penicillin (about 0.5 unit per ml.) is similarly treated. Each dilution is run into a slide cell, sealed with paraffin, and incubated. The highest dilution in both series in which growth is inhibited is determined by inspection. The staphylococci grow into visible colonies in the serum mixtures in which growth is not inhibited. From this the concentration of penicillin in the serum can be calculated.

If adequate amounts of blood can readily be obtained the assay can be carried out more conveniently by using larger volumes of material in small culture tubes, as in the serial dilution method above. For this purpose a standard strain of hemolytic streptococcus may be used as test organism and the amount of growth estimated by the degree of hemolysis produced in a blood-broth culture. The following method* has been used satisfactorily in the Biological Laboratories of the Johns Hopkins Hospital, but this requires precise manipulation and is impracticable for small laboratories making only occasional tests. Fleming has described a micromethod based on this principle, using capillary tubes.

*We are indebted to Dr. G. S. Mirick for the details of this technic.

ASSAY IN BLOOD, USING S. PYOGENES. *Aseptic precautions must be observed in all manipulations.*

MATERIALS NEEDED:

1. Difco beef heart infusion broth, to which has been added 1 per cent Pfanstiel's peptone. This is autoclaved 15 minutes at 15 pounds pressure.

2. Stock culture of *S. pyogenes,* strain C 203. Stock cultures are carried on agar streaked with sterile rabbit blood; they are stored in the icebox and subcultured once a month.

A blood-broth culture is also maintained and stored in the icebox. On the day of the test, 5 ml. of blood-broth are inoculated with 0.1 ml. of the stock blood-broth culture, and used after six to eight hours of incubation. The purity is checked by plating on blood agar.

3. Sterile human group O blood, which is defibrinated and stored in the icebox. This may be used for one week.

4. Penicillin. A 1 : 1000 dilution is prepared as a stock solution. For use this is diluted in broth to 1 : 40,000,000 (final dilution), tubed in 5-ml. amounts, and stored in the freezing (not CO_2) icebox.

5. A sterile specimen of each blood to be tested is ringed with a sterile applicator and placed in the icebox for two hours until it has clotted. It is then centrifuged, the serum removed and stored in the freezing (not CO_2) icebox.

6. Immediately before use, to a given amount of broth are added 4 per cent of the group O blood and 0.4 per cent of a 1 : 10 dilution of the six- to eight-hour streptococcus culture.

SPOT TEST. Into each of three Wassermann tubes are placed (respectively) 1 ml. of a 1 : 10, 1 : 100, and 1 : 1000 dilution in broth of the serum to be tested. To each is added 0.5 ml. of the culture-blood broth mixture. The tubes are incubated at 37° C. for six to eight hours, and allowed to stand overnight at room temperature. They are examined, without shaking, for hemolysis.

FINAL TEST. The highest dilution of serum which completely inhibits hemolysis in the spot test is used, or undiluted serum if none of the three inhibit it. A series of nine sterile tubes is set up for each serum to be tested and one for the penicillin control. Into these tubes are placed the ingredients (in ml. quantities) as indicated in the following tabulation. The tubes are incubated and the results read as in the spot test.

Tube number	1	2	3	4	5	6	7	8	9
Serum dilution (or 1 : 40 million penicillin dilution)	1.0	.8	.6	.4	.3	.2	.15	.1	0
Sterile broth	0	.2	.4	.6	.7	.8	.85	.9	1.0
Culture-blood-broth mixture	.5	.5	.5	.5	.5	.5	.5	.5	.5

The amount of penicillin in the smallest volume of serum which completely inhibits hemolysis is equal to the smallest amount of penicillin which inhibits hemolysis in the control series. From this the quantity per ml. of serum may be calculated.

It is obvious that the dilution of the penicillin used must be so chosen that hemolysis is inhibited by the larger quantities but not by the smaller ones, in order to have a satisfactory basis for comparison with the serum tubes. The use of a different culture strain, different media, or minor variations in technic may necessitate using a dilution different from the 1 : 40 million indicated above.

Determination of the Sensitiveness of an Organism to Penicillin. The agar-plate method can be adapted for this purpose, but the following method is recommended.

SERIAL DILUTION METHOD. For each organism to be tested, serial dilutions of penicillin in meat extract broth (or suitably enriched media, if necessary) are prepared. These should include dilutions containing from 0.25 to 2 units per ml. and larger amounts also

if partially resistant strains are anticipated. One series should contain standard *S. aureus* as a control. Each tube in a series is inoculated with the appropriate organism, incubated overnight, and inspected for growth. This indicates directly the minimum concentration of penicillin necessary to inhibit the organism. This is compared with the standard strain of *S. aureus*.

The determination may be of great practical importance in such infections as bacterial endocarditis caused by *Streptococcus viridans,* since different strains vary considerably in sensitivity to penicillin. Clinically it has been found necessary, as a rule, to secure a concentration in the plasma at least as great as that required to inhibit the strain *in vitro* and preferably two to four times this quantity, in order to eliminate the infection permanently.

Penicillinase. Many penicillin-resistant organisms (and at least one which is sensitive) produce an enzyme-like substance which destroys penicillin. An active preparation can readily be obtained by Berkefeld filtration of a week-old broth culture of a penicillinase-producing organism (e.g., most coliform bacilli), or more conveniently a standardized preparation may be purchased. This is of practical value in the laboratory if cultures are to be made of the blood, body fluids, or secretions of a patient who is being treated with penicillin. The addition of 10 "units" of penicillinase to 15 ml. culture medium makes possible the growth of penicillin-sensitive organisms which might otherwise be inhibited by the penicillin in the material cultured.

PART II

Hematology

By Paul W. Clough, M.D.

Chapters 13–15

CHAPTER 13

Technic of Clinical Blood Examinations

For a study of the cells, either capillary or venous blood may be used. For the preparation of films capillary blood is much preferable. For most other purposes the use of oxalated venous blood is equally satisfactory, and it offers so many practical advantages that many use it routinely in all cases in which a complete examination is to be made. Two ml. suffice for all the ordinary tests.

Venous Blood. To obtain accurate results with venous blood, the following precautions must be observed. The syringe and needle must be dry, or rinsed out with sterile physiological salt solution. More than a very brief period of stasis must be avoided or the counts will be too high. If a tourniquet is used, it must be released as soon as the needle enters the vein, and one must wait until normal conditions are restored before filling the syringe. A definite amount of anticoagulant must be used. Heparin (1 mg. per 5 ml. blood) is the best, except that it clumps the leukocytes. Potassium oxalate has generally been employed. If one works quickly, 2 mg. per ml. of blood suffice, and are preferable to larger amounts. If chemical examinations are not to be made on the same specimen, however, it is better to substitute for potassium oxalate a *mixture of 6 parts of ammonium oxalate and 4 parts of potassium oxalate,* since this does not cause shrinkage of the red cells. We recommend preparing oxalate tubes as follows: To each of a series of small test tubes add with a graduated pipet 0.04 ml. of a solution containing 6 per cent ammonium oxalate and 4 per cent potassium oxalate. Allow the fluid to evaporate in the incubator or drying oven at low temperature, and stopper. When needed, add just 2 ml. blood from a syringe, and mix thoroughly at once.

Except for platelet counts and films, which must be made at once, the examinations may be postponed for three hours (for ordinary cell counts 24 hours). More time can be taken in filling the pipets and greater precision attained with venous blood than with capillary blood, and duplicate determinations can easily be made if necessary. Immediately before filling the pipets the blood must be thoroughly mixed, preferably by inverting the tube repeatedly and gently, so as to avoid frothing.

Capillary Blood. Capillary blood may be obtained either from the ear or finger, or, in infants, from the great toe or heel. The finger is preferable as it is easier to secure uniform satisfactory circulatory conditions there than in the ear. If the patient objects to the use of the finger tip, the area on the dorsum of the finger between the nail and the first joint may be used. The part must be warm and hyperemic. If cold or cyanotic, the hand should first be immersed in warm water, or the ear lobe vigorously massaged. The skin is cleaned with 70 per cent alcohol and dried. An abrupt quick stab to a depth of about 2 mm. is made with a blood lancet or some substitute instrument with a short, sharp cutting edge. We prefer a lancet of the spring-release type with a screw by means of which the depth of the puncture can be precisely adjusted. A good substitute is a Hagedorn needle, or a steel pen with one nib broken off, and the other sharpened on a fine-grained whetstone. Wright's glass needle may be used. The lancet is sterilized by dipping in alcohol and drying, or flaming. A freely flowing drop is essential, although a little gentle tension on the sides of the finger to separate the margins of the puncture wound

is occasionally necessary and permissible. Greater pressure causes stasis and concentration of the blood. Violent squeezing may dilute the blood with tissue juice. The first drop or two is wiped away, and subsequent drops used for the examinations. If properly obtained, capillary blood and oxalated venous blood yield identical results except in macrocytic anemias, in which the red cell count may be higher in capillary blood than in venous blood.

Routine Blood Examinations. For the average patient it often suffices to make a hemoglobin (or red cell volume) determination, a leukocyte count, and a survey of a stained film. If any abnormality in the blood is found or suspected, the examination should include also a count of the red cells, a determination of the sedimentation rate and cell volume, and a differential count. If there is anemia, the icterus index and reticulocyte count should be included, as well as any other special examinations which may be indicated in the particular patient being studied.

Fresh Blood Preparations. Microscopic examination of a fresh drop of blood is useful as a preliminary procedure, and if this shows no abnormality it is often possible to dispense with further examinations. A freely flowing drop of blood about 2 mm. in diameter is brought in contact with the middle of a clean coverslip. The specimen is mounted immediately on a clean glass slide, the coverslip being tilted slightly as it is lowered to prevent formation of air bubbles. The blood should spread out under the weight of the coverslip in a uniform layer, without any pressure. The coverslip is sealed with petroleum jelly. The blood is examined under the oil-immersion lens with dim illumination, care being taken not to strike the coverslip with the objective, or the cells will be crushed. In the center of the preparation the red cells should be evenly distributed in a single layer, not piled up or arranged in rouleaux, and not distorted. With some experience any abnormality in the size, shape, or depth of color of the cells can be detected at once. The characteristic changes in sickle cell anemia can be made out only in fresh preparations. A leukocytosis or leukopenia can be recognized, as well as any notable abnormality in the differential count. Such preparations are useful in the study of malarial and other parasites.

Hemoglobin Estimation

There is no method available for the direct estimation of hemoglobin. The most important function of hemoglobin, and, as far as known, its most constant property, is its ability to combine with oxygen, in the proportion of one molecule of oxygen to each atom of iron that it contains. Since the weight of hemoglobin cannot be determined directly, the most logical and actually the most precise method of estimating the hemoglobin content of the blood is to determine its *oxygen-combining capacity*. This determination offers no great difficulty to the trained laboratory worker, but it is entirely impracticable as a routine clinical procedure. It is of fundamental importance, however, in checking the accuracy of other methods, and in the standardization of hemoglobinometers. (For technic see p. 809.) This procedure measures only the oxyhemoglobin and reduced hemoglobin. If any of the hemoglobin is present in other forms, such as methemoglobin or sulfhemoglobin, this portion will be missed, whereas it is included in the usual colorimetric determinations.

All practicable methods of hemoglobin estimation are *colorimetric*. To eliminate the misunderstandings due to the many different standards of normal which have been proposed by various observers, recent workers have urged that the custom of expressing the amount of hemoglobin as a percentage of normal be abandoned, and that instead instruments be calibrated in grams of hemoglobin per 100 ml. of

blood. Most modern instruments are so calibrated. Although it has been generally accepted that 1 Gm. of hemoglobin has an oxygen-combining capacity of 1.34 ml., attempts to determine this factor accurately meet with serious technical difficulties, and the figure 1.34 is probably only roughly approximate at best. Although the present custom is an improvement over the previous ones, merely expressing hemoglobin in grams per 100 ml. does not eliminate errors in its determination. Since many clinicians still prefer to think of hemoglobin in percentages of normal, it is desirable to calculate the latter figure also, by dividing the reading in grams by the number of grams of hemoglobin in the accepted normal standard.

Sahli's Hemometer. We regard this instrument, or one of the recent modifications, as the most practicable for routine clinical use. The original type of instrument utilizes as a standard a sealed tube containing a suspension of acid hematin corresponding to a 1 per cent dilution of normal blood. A glass bead is included to facilitate mixing. The blood is diluted in an open tube of the same diameter so calibrated (in per cent, or in the newer instruments in grams per 100 ml.) that when filled to the 100 per cent mark it holds just 2 ml.

Procedure. (1) Invert the standard tube several times to mix. (2) To the open tube add N/10 HCl to the 10 mark (or higher). (3) From a freely flowing drop of blood, or well-mixed oxalated blood, fill the special capillary pipet provided just to the mark (20 cu. mm.) by gentle suction. (4) *Thoroughly* wipe off all the blood adhering to the outside of the pipet. (5) If the blood has been drawn slightly beyond the mark, bring it back to the mark by stroking the tip of the pipet with the finger. (6) Expel the blood into the acid solution in the tube, rinse the pipet with the solution, and mix the contents of the tube thoroughly. (7) Allow the mixture to stand (not in a bright light) for 10 minutes or longer. (8) Dilute with N/10 HCl (or distilled water) until the color exactly matches that of the standard tube. Add one drop at a time as the end point is approached, and mix the contents of the tube well with a fine glass rod after each addition. If the tube is closed with the finger tip and inverted, some of the fluid will be lost. (9) Read the level of the fluid in the diluting tube and multiply this figure by the correction factor of the instrument. (10) Rinse and dry the pipet and tube at once, and put the instrument away out of the light.

The development of the brown color is a gradual process, proceeding rapidly at first, then more slowly. After 10 minutes about 96 per cent of the maximum color has developed, and after an hour, practically all. The method chosen should be adhered to consistently and should be used in the standardization of the instrument. If the instrument is standardized for a 10-minute interval and the dilution is materially delayed, 2 per cent should be subtracted from the corrected reading.

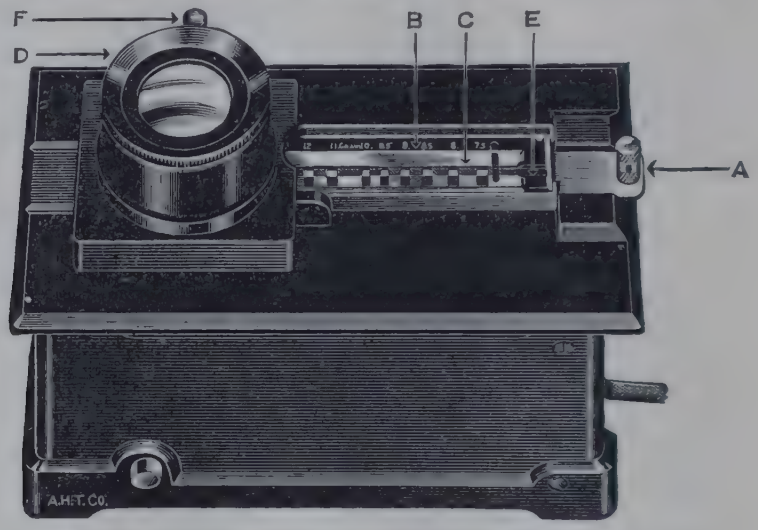
The advantage in the use of this instrument is that, as the solutions being compared are chemically identical, accurate readings can be obtained with any reasonably bright light. The great disadvantage is that the standard tube fades, rapidly if the instrument is carelessly exposed to sunlight. Frequent restandardization is therefore imperative, at least once in three months, if gross errors are to be avoided.

To obviate this difficulty, the newer instruments are provided with tinted glass rods which do not fade, in place of the standard tube of acid hematin suspension. The *Sahli-Hellige hemometer* is one of the most satisfactory of these modifications. The color standard consists of a pair of four-sided glass prisms, between which is mounted the dilution chamber which is made with four plane surfaces. On one side this is graduated in grams per 100 ml., and on the opposite side in per cent, 14.5 Gm. being adopted as 100 per cent. The procedure is exactly the same as with the Sahli instrument, except, as with all instruments using colored-glass standards, *a uniform source of light must be used,*

preferably a colorimeter lamp provided with a daylight-glass window. The reading of the same blood by daylight may be 10 per cent lower than by an ordinary Mazda light. The initial standardization should not be omitted, but once obtained the correction factor does not change.

Numerous attempts have been made to find a colored fluid which would be a satisfactory substitute for the acid hematin standard solution in the original Sahli instrument, but all have either faded or deepened in color with use.

Haden-Hausser Hemoglobinometer. The blood is diluted 1 : 20 with N/10 HCl in a hemacytometer pipet for white corpuscles (1 : 10 if the hemoglobin is less than 7.5 Gm. per cent). This is introduced into a wedge-shaped chamber in a glass comparator slide and compared with the color of a strip of standard glass of uniform thickness incorporated in the slide in immediate juxtaposition to the chamber. The comparator slide (B) is covered with a rectangular coverglass (C) and carried in a metal holder (A)



Haden-Hausser hemoglobinometer, laboratory model.

which is moved laterally across the stage of the instrument. It is viewed through a reading microscope (D), using for illumination a small electric-light bulb with a Daylite-glass filter. There appears in the field of the microscope a series of illuminated rectangles (15 in all, corresponding to hemoglobin concentrations of 7.5 to 19 Gm.) separated by dark spaces. The lower half of the rectangles is occupied by the standard colored-glass strip, and the upper half by the wedge-shaped chamber of varying depth containing the diluted blood. The comparator slide is moved back and forth until the rectangle which shows identical color in both upper and lower halves is in the center of the field. By moving a shutter (F), the scale readings which are invisible while the comparison is being made are brought into the field and the hemoglobin concentration in Gm. % is read directly.

The instruments have been carefully standardized by means of blood whose oxygen-carrying capacity has been determined by Van Slyke's method. It is desirable that this be checked in the laboratory before use. With due care and a proper light, accurate results can be obtained.

Procedure. (1) Mount the comparator slide in the holder; apply the coverglass after brushing off any dust or lint from its surface, and insert the slide in the channel on the top of the bakelite case. (2) Draw blood (oxalated or from a finger) to the 0.5 mark of a white-blood-cell diluting pipet (to the 1.0 mark in case of notable anemia), and fill the pipet to the 11.0 mark with N/10 HCl. (3) Mix the contents of the pipet gently and let stand for 30 minutes or longer. (4) Shake the pipet and discard the fluid in the capillary portion. (5) Discharge the remainder of the diluted blood into the wedge-shaped chamber of the comparator at the end of the coverglass (E). (A very thin uniform film of diluted blood is drawn by capillary attraction over the surface of the colored-glass

standard. This is advantageous as it insures a uniform light-transmitting surface over both the standard and the diluted blood.) (6) Move the comparator slide across the field until a satisfactory match is obtained. (7) Open the shutter and record the reading. To avoid errors due to retinal fatigue it is desirable to rest the eyes two or three minutes and check the reading. (8) Clean and dry the chamber and coverglass immediately.

If the blood has been diluted 1 : 10, divide the reading by 2. To express in per cent, divide the reading by 14.5 (or by whatever figure has been selected as the normal standard of 100 per cent).

Photoelectric Colorimeter. A glass disc of a color corresponding to a specified concentration of hemoglobin is commonly used as a standard. (1) In a colorimeter tube put exactly 5 ml. of N/10 HCl. (2) Fill a Sahli hemoglobinometer pipet to the 20-cu. mm. mark with blood (either oxalated or from a finger puncture). (3) Wipe all blood from the outside of the pipet, expel the blood into the acid, rinse the pipet with the solution, and mix the contents of the tube thoroughly. (4) After one hour (or more) read in the colorimeter, and also make a reading with the standard disc.

Calculation:

$$\text{Hb. in Gm. \%} = \frac{\text{Reading of unknown} \times \text{corrected value of standard disc}}{\text{Reading of standard disc}}$$

The accuracy of the standard should be checked, using blood of known hemoglobin content, as directed in the following paragraph (Standardization). If this precaution is taken, accurate, closely reproducible estimations can be readily obtained.

Standardization. (A) (1) Obtain oxalated venous blood from a healthy young man with a normal red-cell count, and determine carefully the hemoglobin reading (in grams) on the instrument to be tested, taking the average of several observations. (2) Determine the oxygen-combining capacity of the same blood in volumes per cent, and calculate the hemoglobin content in grams by dividing this figure by 1.34. If this is not practicable, determine the hemoglobin with some other instrument which has been so standardized and is known to be accurate. (3) Determine the correction factor by dividing the *correct* reading by the reading on the instrument being tested.

For example, if the correct reading is 15 Gm., and the reading on the instrument to be tested is 14 Gm., the standard of the latter is too dark. The correction factor is $^{15}/_{14}$, or 1.07. If the correct reading is 15 Gm. and the reading on the instrument is 17 Gm., the standard is too pale; the correction factor is $^{15}/_{17}$, or 0.88.

The total iron content of the blood (Wong's method) may be used for standardization if it is impracticable to determine the oxygen-combining capacity. This is less accurate, as the blood contains a small amount of iron not combined in hemoglobin.

(B) If neither procedure is possible: (1) Make a careful red-cell count on a specimen of normal oxalated blood. It is preferable to use pooled blood from several different individuals. The count must be at least 5.0 million. (2) Dilute the suspension with sufficient physiological salt solution to bring the count just to 5.0 million. It is assumed that this contains just 14.5 Gm. hemoglobin per 100 ml. (3) Carefully determine the hemoglobin of this adjusted blood with the instrument to be tested. (4) The factor is determined by dividing 14.5 by the reading. Although this procedure does not give research accuracy, it avoids any error of clinical significance.

The minimum probable error, if the instrument has been accurately standardized, is about 2 per cent and in actual practice is often nearer 5 per cent.

Tallqvist's Hemoglobin Scale. This has been much used because of its great simplicity. Some of the newer improved color scales give reasonably accurate reproducible readings, although less precise than those obtainable with the better instruments. They are less reliable in cases of severe anemia. The following precautions should be observed. The drop of blood should be large enough to make a stain at least 1 cm. in diameter. Readings should be made in diffuse daylight, as soon as the spot loses its gloss. They are best made

by cutting out the spot and putting it over the color-scale plates. The colors of the standard plates fade if exposed to light.

Determination of Iron Content (Wong)

Procedure. (1) With a volumetric pipet place 0.5 ml. blood in a 50-ml. volumetric flask. (2) To this add 2 ml. concentrated sulfuric acid, and mix by whirling the flask for one or two minutes. (3) Warm the flask if necessary, add 2 ml. saturated potassium persulfate solution and mix by shaking. (4) Dilute to about 25 ml. with distilled water, add 2 ml. of 10 per cent sodium tungstate solution and mix. (5) Cool under the tap, dilute to volume with distilled water, stopper, mix and filter. (6) Pipet exactly 20 ml. filtrate into a test tube graduated at 20 ml. and 25 ml. (7) Into a similar tube pipet 1 ml. of standard solution containing 0.1 mg. iron. (8) With a serologic pipet add 0.8 ml. concentrated sulfuric acid to the standard solution. (9) Dilute to 20 ml. with distilled water and cool under the tap. (10) To both tubes add 1 ml. saturated potassium persulfate solution and (11) 4 ml. of 3 N potassium sulfocyanate solution. (12) Stopper both tubes, mix, and read in the colorimeter.

Calculation with a photoelectric colorimeter:

$$\text{Mg. of iron per 100 ml.} = \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times \frac{0.1 \times 100}{0.2}, \text{ that is, } 50$$

Calculation with a Duboscq type of colorimeter:

$$\text{Mg. of iron per 100 ml.} = \frac{\text{Reading of standard}}{\text{Reading of unknown}} \times 50$$

To determine the hemoglobin per 100 ml. blood, this figure is divided by 3.35.

Reagents. These must be free from iron. This is checked by running a blank determination on the reagents, and a correction factor is established if necessary.

Sulfuric Acid, Concentrated.

Sodium Tungstate. A 10 per cent solution, as used in preparation of blood filtrates, is used.

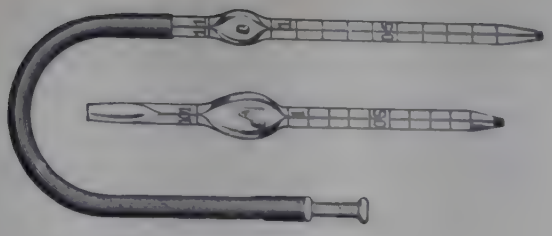
Potassium Persulfate, Saturated Solution. About 7 Gm. potassium persulfate and 100 ml. distilled water are placed in a glass-stoppered bottle and mixed by shaking. Some excess of crystals should be present. The solution should be renewed frequently.

Potassium Sulfocyanate, 3 N Solution. One hundred forty-six Gm. potassium cyanate are dissolved in sufficient distilled water to make 500 ml. The solution is filtered and 20 ml. pure acetone are added as a preservative. This need not be precisely accurate.

Standard Iron Solution. Exactly 0.861 Gm. crystallized ferric ammonium sulfate is placed in a 1-liter volumetric flask and dissolved in about 50 ml. distilled water. Twenty ml. concentrated sulfuric acid are added and the solution is then diluted to the mark. One ml. = 0.1 mg. iron. If determinations are made in cases of anemia, weaker standards should be prepared by diluting the stock standard solution.

Enumeration of the Blood Corpuscles

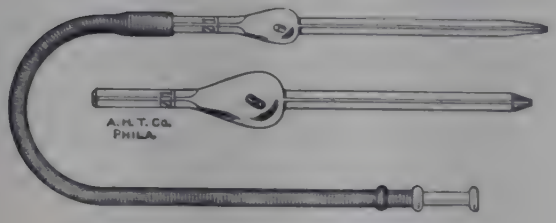
Hemacytometer. We recommend the Levy-Hausser type of counting chamber with a double platform, and with the "improved" Neubauer ruling. The counting chamber is made of a single solid piece of glass, and is carried in a Bakelite holder which can be used conveniently on a microscope stage, and which protects the chamber from scratching and breakage. In the improved Neubauer ruling the central square millimeter is divided, as in previous types, into 400 smallest squares. The latter are arranged in 25 groups of 16 squares each by making each fifth vertical and horizontal line a double line, or in the most recent model, a split line. For diluting the blood the ordinary Thoma diluting pipet



Thoma diluting pipets.



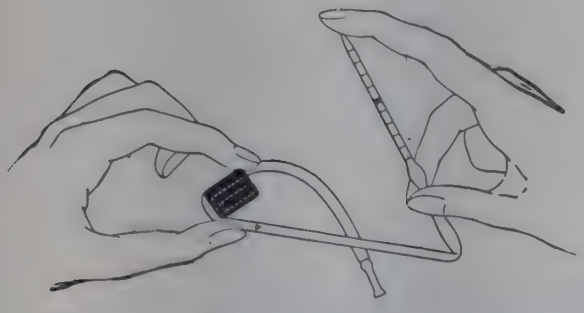
Thoma diluting pipet bulb enlarged.



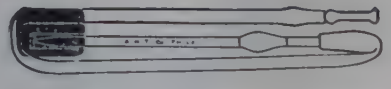
Trenner automatic diluting pipets.



Trenner diluting pipet bulb enlarged.



Closure for blood diluting pipets showing closure in position on rubber tubing, filled pipet closed with finger tip, and rubber tubing sharply kinked prior to rotating tip into position in closure.



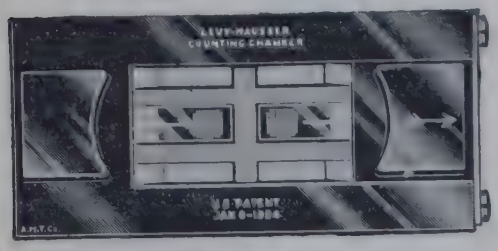
Closure for blood diluting pipets showing pipet tip in position in closure. Pipet is now automatically sealed.

is satisfactory. For those who have difficulty in drawing the blood exactly to the mark on the stem of the Thoma pipet, the Trenner diluting pipet is recommended. The hard-rubber closure illustrated is convenient to prevent leakage and facilitate handling of the pipet while it is being shaken. It is desirable to use apparatus which has been certified by the Bureau of Standards.

Enumeration of the Red Corpuscles. Procedure. The tip of the diluting pipet for red cells, carrying the graduation 101 on the stem above the bulb, is applied to a fairly large drop of blood; and with the pipet in a horizontal position, blood is drawn by gentle suction just to the 0.5 mark (or to the 1.0 mark if there is marked anemia). If the column of blood goes slightly past the mark, it may be brought back to the line by gently tapping the tip of the pipet on the finger or on a piece of filter paper. If the Trenner pipet is used, the blood is similarly drawn in until the stem is nearly filled. It is then allowed to flow in by capillary attraction (without suction) until the bore is filled, when it automatically stops. Any blood on the outside of the pipet is quickly wiped off, the tip is inserted in the diluting fluid, and the bulb of the pipet filled by gentle suction until the fluid reaches the 101 mark on the stem above the bulb. To avoid air bubbles the pipet is held vertically; the pipet is twirled between the fingers while it is being filled, and, if the glass bead sticks to the glass, it is dislodged by tapping with the finger. The tip of the pipet is closed at once with the finger or rubber closure, and the pipet shaken a few seconds to ensure mixing. Up to this point, if capillary blood is being used, one must work rapidly to prevent clotting. The pipet may now be set aside for a few hours before completing the count.

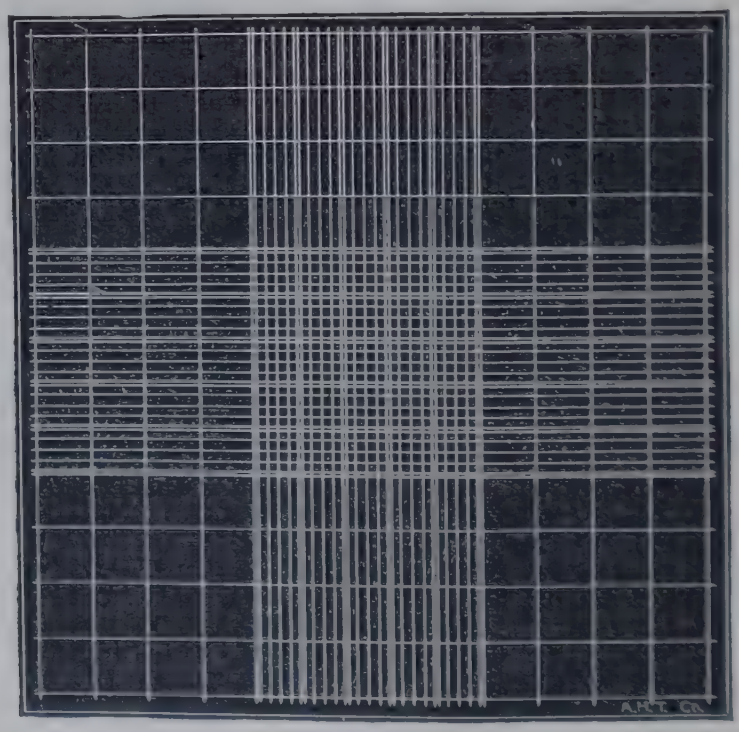
The counting chamber is placed on a level surface, dust is brushed off with a camel's-

hair brush, and the coverslip is applied. Both must be perfectly clean. If a certified coverslip is used, it must be applied with the Bureau of Standards mark uppermost. The pipet is shaken for about two minutes, the pipet being held horizontally and shaken at right angles to its long axis. Two or three drops are blown out of the pipet and the tip is applied to the top of the ruled platform of the counting chamber at the edge of the coverslip. While blowing very gently if necessary, enough of the diluted blood is allowed to flow out to cover the platform completely but not to overflow into the moat. Air bubbles must be avoided. The other platform is then covered in a similar manner. The counting chamber is inspected under the low-power lens of the microscope. If the cells are not evenly distributed, or if the fluid overflows into the moat, the chamber is cleaned, the pipet reshaken, and the procedure repeated until a satisfactory distribution is obtained. The chamber should be allowed to stand a few minutes until the cells have settled.



Levy-Hausser counting chamber.

With the high-power dry lens the red cells in five of the groups of 16 smallest squares are counted. To get the most representative distribution it is best to count the four corner groups and the central group. A mechanical counter greatly facilitates the counting. To avoid counting the same cell twice, cells which touch a line are counted if they touch the left hand or upper line of a square, and are not counted if they touch the right hand or lower line, regardless of where the bulk of the cell may lie. We prefer this method, even



Entire area of improved Neubauer ruling showing split boundary lines (400 small squares available for counting.)

with the newer types of ruling, and regard a cell as "touching" a line if it is in contact with any part of the double or split line. Any white cells which are present are also counted. If the leukocyte count is significantly high it may later be subtracted from the total count. The cells on the second platform are then counted and the average of the two counts is taken. The counts should not differ by more than 20 or at most 30 cells.

Calculation. The cells have been counted in 5 groups of 16 smallest squares or in 80 of the 400 squares, that is, in $\frac{1}{5}$ of the sq. mm. The depth of the chamber is $\frac{1}{10}$ mm. The

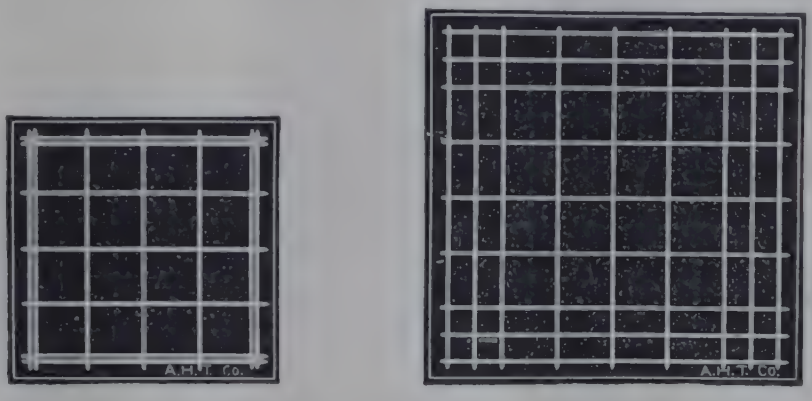
dilution of the blood is (usually) 1 : 200. The total red cell count per cu. mm. is therefore obtained by multiplying the number of cells counted (average of the two fields) by 10,000 (5 × 10 × 200).

The probable error, with due care and experience, is at least ± 4 per cent, or 200,000 cells in a total count of 5,000,000. The expert can reduce this only by taking the average of repeated determinations.

Diluting Solutions. Hayem's solution is recommended. (Sodium chloride, 1.0 Gm.; sodium sulfate, 5.0 Gm.; mercuric chloride, 0.5 Gm.; water, 200 ml. This should be filtered occasionally.)

Toisson's solution is satisfactory. (Sodium chloride, 1.0 Gm.; sodium sulfate, 8.0 Gm.; glycerin, 30 ml.; water, 160 ml.)

In an emergency 1 per cent sodium chloride solution may be used, but it is less satisfactory.



(*Left*) Group of 16 smallest squares of improved Neubauer ruling showing split boundary lines. Entire group visible simultaneously with 4-mm. objective.

(*Right*) Group of 16 smallest squares of original Neubauer ruling showing boundary by fifth squares with extra line in middle of each. The border squares necessitate readjustment of focus.

Enumeration of the White Corpuscles. Using the Thoma (or Trenner) diluting pipet for white corpuscles with the graduation 11 on the stem above the bulb, blood is drawn up to the 0.5 mark (to 1.0 if there is a leukopenia), and then diluting fluid to the 11 mark, just as directed for counting red cells. As diluting fluid we prefer Türk's solution (glacial acetic acid, 1.0 ml.; 1 per cent aqueous gentian violet solution, 1 ml.; water, 100 ml.; the solution should be filtered frequently to remove yeasts that may grow in it.)

Shake the pipet and cover the two platforms of the counting chamber with diluted blood, as in counting red cells. Make certain that the cells are evenly distributed by inspecting the entire ruled area under the low-power objective. Count the total number of leukocytes on 5 of the 9 sq. mm. areas on each of the two platforms. With some experience this can be done with the low-power objective, although beginners may mistake yeast cells or small pieces of dirt for leukocytes, unless the high dry objective is used.

Calculation. The total area counted is 10 sq. mm. The depth of the chamber is $\frac{1}{10}$ mm. The cells from 1 cu. mm. of diluted blood have therefore been counted. The total leukocyte count per cu. mm. is obtained by multiplying the total number of cells counted on both platforms by the dilution of the blood, usually 20. If the leukocyte count is very high, as in leukemia, the blood should be diluted 1 : 100 or 1 : 200 in a red cell pipet with Türk's diluting fluid; or the cells may be counted in all or a portion of the central sq. mm., as in counting red cells. The calculation must be altered accordingly. Nucleated red cells, if present, are included in the count. The probable error is greater than in the enumeration of red cells, since as a rule fewer leukocytes are actually counted.

Enumeration of the Blood Platelets

Many methods have been suggested, none of which are entirely satisfactory.

Direct Method. (1) Moisten the inside of a Thoma diluting pipet for red corpuscles with diluting fluid by aspirating a little fluid into the pipet and expelling the fluid. (2) Fill the stem of the pipet to the 1.0 mark from a freely flowing drop of blood. (3) Fill the pipet with diluting fluid. (4) Shake at once, and place a drop of the diluted blood on each platform of the counting chamber. (5) Place the counting chamber in a moist chamber on a level surface, and allow it to stand 15 minutes, until the platelets have settled. (6) With the high-power dry objective count the number of platelets in 5 groups of 16 smallest squares in each platform. (7) Multiply the average count of the two fields by 5000.

As diluting fluid one may use:

1. Sodium citrate, 3.5 per cent solution.
2. Wright and Kinnicutt's solution: Of a 1 : 300 solution of brilliant cresyl blue, 2 parts; of a 1 : 1400 solution of potassium cyanide, 3 parts.
3. Rees and Ecker's solution: Sodium citrate, 3.8 Gm.; formaldehyde, neutral 40 per cent, 0.2 ml.; brilliant cresyl blue, 0.1 Gm.; water, 100 ml. This is stored in an icebox in a glass-stoppered bottle and renewed frequently.
4. Howell and Donahue recommend: Sodium chloride, 2 per cent solution, 50 ml.; potassium phosphate (11.876 Gm. per liter), 40 ml.; potassium hypophosphate (9.078 Gm. per liter), 10 ml.; heparin, 60 mg. This should be freshly prepared or kept in the icebox.

Just before use, all these solutions should be filtered and centrifuged at high speed to remove bacteria and other foreign particles which seriously interfere with the accuracy of the counts.

Counts obtained by these methods are lower (200,000 to 400,000 in normal blood) than by the usual indirect methods, which tend to concentrate the platelets. The probable error is high, about 15 to 20 per cent.

For practical purposes anyone with a little experience can estimate the number of platelets with sufficient accuracy by inspecting a well-made coverslip (stained) film (normally there is one platelet to each 7 to 15 red cells). This is always desirable as a check on the accuracy of a count.

Volume of the Red Corpuscles

We recommend the hematocrit tube of Wintrobe. By means of a rubber-bulb pipet with a long, slender tip the tube is filled to the 10.0-cm. mark with well-mixed oxalated blood. To avoid air bubbles, the tip of the pipet should be inserted to the bottom of the tube, and then be gradually withdrawn as blood is expelled into the tube. The sedimentation rate may first be obtained. The tube is then centrifuged in an ordinary holder until the cells are sedimented to a constant volume. A good electric centrifuge is required, capable of a speed of at least 2500 revolutions per minute. The time required is usually 20 to 30 minutes, but must be determined for the centrifuge. Prolonging centrifugation does not compensate for inadequate speed. The chief source of error is inadequate centrifugation, which gives too high a reading. After packing of the cells is complete, the volume of red cells in per cent is read directly from the graduation at the top of the column of red cells. This must be corrected for shrinkage of the cells if potassium oxalate is used. It is multiplied by 1.04 if 2 mg. solid potassium oxalate per ml. of blood was used.

Van Allen's hematocrit tube may be used to determine the cell volume with capillary blood. Blood is drawn by suction just to the 100 mark. The anticoagulant, an isotonic (1.3 per cent) solution of sodium oxalate, is then drawn up into the tube until the bulb is about one-third filled. The exact amount is immaterial. The tube is then sealed by draw-

ing a stout rubber band over both ends of the tube, or preferably by one of the spring clips furnished with the instrument. It is then centrifuged until the cells are packed to a constant volume, and the graduation at the top of the cell column gives the reading directly in per cent. No correction for shrinkage is necessary. It is less satisfactory than the Wintrobe tube, if venous blood is available.

Preparation of Blood Films

Films may be made on slides, or on coverslips. For differential leukocyte counts coverslips are much to be preferred, as the leukocytes are more evenly distributed. In films spread on slides the large cells tend to accumulate at the margins and end of the film, whereas the lymphocytes are largely scattered through the middle of the film. Slides are satisfactory for the examination of red cells, for the study of individual leukocytes, and for search for parasites. They are preferable for thick-film preparations.

Cleaning Glassware. Coverslips should be 22 mm. square, No. 1 or No. 2, of the best grade obtainable. Coverslips and slides used for making blood films must be absolutely clean and free from grease. To clean coverslips we prefer to drop the slips individually into sulfuric acid-potassium bichromate cleaning solution and let them remain in it 24 hours or longer. The slips are then removed, rinsed individually in running water, then in distilled water, and dropped into 95 per cent alcohol. To dry them conveniently, a thin, smooth piece of board about 8 inches square, padded well and wrapped tightly with a clean linen towel, is used. With coverslip forceps the slips are removed from the alcohol, spread singly on the padded board, and dried by rubbing them gently with a handkerchief (first scrubbing the hands to get them free from grease). The slips are picked up with forceps and stored in a dust-proof box. Glassware that is to be used for blood films should never be handled with the fingers. Some prefer to polish the slips with jewelers' rouge, but we have not found this necessary.

Slides may be cleaned in the same way. They may be cleaned by scrubbing with soap and water, rinsing thoroughly, drying, and passing through the flame of a Bunsen burner to remove the last trace of grease. More satisfactory results can be obtained by using Bon Ami. Some of the Bon Ami is rubbed up with the wet finger, the slide is rubbed with the lather until there is a friction squeak; it is allowed to dry and is polished with a clean dry cloth. The advantage of using such substances as jewelers' rouge, Bon Ami, and strong acids is that they not only remove grease, but probably slightly etch the surface of the glass, and facilitate even spreading of the film.

Coverslip Films. With forceps remove several coverslips from the stock box, and brush off any lint or dust with a clean camel's-hair brush. Touch a small freely-flowing drop of blood about 2 to 3 mm. in diameter with the center of a clean coverslip, taking care not to let the slip touch the skin. Lower this immediately onto a second coverslip, allowing the weight of the coverslip to spread the drop of blood. No pressure is permissible. When the spreading of the drop begins to slow down, grasp the upper coverslip again and pull it away from the other in such a way that the plane surfaces of the two slips are perfectly parallel throughout the manipulation. Any tilting of the upper coverslip away from the lower will ruin the preparation. The coverslips, during the manipulation, may be handled with forceps or with the fingers, as the operator prefers. The films are dried in air, and must be protected from dust and insects.

Films on Slides. A freely flowing drop of blood about 3 or 4 mm. in diameter is touched with a clean glass slide at a spot about one-half inch from the end of the slide. The slide is placed on the table, and the end of a second "spreader" slide is lowered onto the first slide, slightly in advance of the drop, so as to make an angle of about 30 degrees with the first slide. The spreader is drawn back until the edge touches the drop. As soon as the blood runs out along the line of contact, the spreader slide is advanced with a quick, even sweep to the other end of the slide, so that the blood is pulled (not pushed) along behind the edge of the advancing spreader. It is preferable to narrow the spreader

slide by filing off a triangular piece about ⅜ inch wide at one corner. This makes the film narrower than the slide and facilitates examination of the cells along the margin of the film. By increasing the angle between the two slides thicker films may be obtained and vice versa. In place of the slide a strip of cigarette paper may be used for a spreader, and gives better results in unskilled hands.

Staining and Examination of Blood Films

For routine purposes some modification of the Romanowsky polychrome methylene-blue stain is almost universally used. Of these we regard Giemsa's stain as the best. However, the stain is expensive, and considerable time is required for staining the films. For these reasons a simpler type of stain is usually employed. We recommend Wright's stain, which is most generally used in this country, or Wilson's stain, which gives practically identical preparations. Wilson's stain is much better for the demonstration of blood platelets. We recommend the purchase of these stains in solution ready for use, or in the form of dried powder or tablets, to be dissolved in methyl alcohol when needed. The methyl alcohol must be of the highest purity, anhydrous, and free from acetone.

Technic of Staining. For the best results the (air-dried) films should be stained within 24 hours. Coverslips are most conveniently handled by using a coverslip forceps, such as the Stewart type, which holds the cover in a perfectly horizontal position. (1) Cover the dry film with the alcoholic solution of the stain for one minute. This fixes the film. (2) Add rapidly an equal quantity of diluent, best with a rubber-bulb pipet, and stain the film for five minutes (or longer if necessary) until a metallic scum forms on the surface. (3) Wash by flooding the film with distilled water or diluent until it has a pinkish tinge. (4) Dry in air by tilting the coverslip on one edge so that the water will drain off, or blot carefully with fine blotting paper.

Trouble from the deposition of a precipitate on the film may be avoided by placing the coverslip with the film down in a small shallow watch glass, and adding with a pipet just enough of the undiluted stain to float the coverslip. After one minute an equal volume of diluent is added, care being taken to keep the coverslip floating on the diluted stain. After five minutes the coverslip is removed with forceps, washed, and dried as above.

The dried film may then be mounted in immersion oil (cedar oil) for examination, or, if it is to be preserved, in special *neutral* Canada balsam (which may be purchased from reliable dealers). To ensure a neutral mounting medium, a relatively dilute, thin solution of balsam damar in xylol is made, powdered calcium oxide is added, the mixture shaken well and allowed to stand several weeks until it clears by sedimentation.

As *diluent* one may use carbon dioxide-free distilled water, or (if the stain is alkaline) the following *buffered phosphate solution:* recrystallized monobasic potassium phosphate, 6.63 Gm.; anhydrous dibasic sodium phosphate, 2.56 Gm. (both of the highest purity); distilled water, 1 liter. This should have a pH of 6.4. To preserve, 1 ml. chloroform is added to the stock bottle.

If these reagents are not available and the distilled water is too acid, the following may be tried: (1) Boil the water to expel carbon dioxide, and cool. Test by adding a bit of hematoxylin to a few ml. in a small test tube. If the color does not turn blue in from one to five minutes, the water is still too acid. Correct this by adding traces of 1 per cent sodium carbonate solution until it becomes blue. (2) To 5 ml. of distilled water which has been boiled and cooled, add a small drop of a 1 per cent aqueous solution of neutral red. If the color turns pink (acid), with a glass rod add successive traces (not drops) of 1 per cent sodium carbonate solution until it turns a pinkish yellow (neutral). This may be used. However, if the color turns to a clear yellow, the water is too alkaline and must be discarded. One must start again with fresh water.

If distilled water is not obtainable, rain water collected in the open is the best substitute.

Giemsa's Stain. Three tenths Gm. of Azur II-eosin and 0.08 Gm. of Azur II are dissolved in 25 ml. of pure anhydrous glycerin at 60° C. Twenty-five ml. of absolute

methyl alcohol are added at the same temperature. The solution is allowed to stand overnight and then filtered. Immediately before use one part of the stain is diluted with 10 to 15 parts of distilled water or buffered solution. If intense staining is desired, the stain is diluted with 1 : 1000 solution of potassium carbonate.

Procedure. The film is fixed in absolute methyl alcohol for one to five minutes, then dried. The coverslip is covered with diluted stain, or, preferably, floated on the diluted stain, and left for 15 to 30 minutes. At the end of this period it is washed in distilled water until the film has a pinkish tinge, then dried, and mounted.

The alkaline diluent is used to demonstrate the coarse stippling in the red cells in malignant tertian malaria, and to stain spirochetes. To demonstrate *Treponema pallidum* the film is stained for 2 to 12 hours.

The *panoptic stain* (or "combined Giemsa") is somewhat superior to the simple Giemsa stain for blood films. The air-dried film is first stained with Jenner's stain (identical with the May-Grünwald stain), or with Wright's stain, as above outlined. The film is washed and without further fixation is counterstained with diluted Giemsa's stain.

Hematoxylin and Eosin. This stain is useful if good preparations of the preceding stains are not available, and is preferred by many for Arneth counts. Films are best fixed by heat, although alcohol may be used.

Heat Fixation. 1. If a drying oven is available, the films are placed in the oven, heated gradually to 150° C., and allowed to cool gradually in the oven.

2. The films (preferably more than 24 hours old) are placed face down on a copper bar, along with a crystal of urea. They are heated gradually over a small flame until the crystal melts (135° C.), then the bar is allowed to cool. Todd advised using a tartaric acid crystal, which melts at about 168° C., and removing the films when the crystal melts. If fresh films are to be fixed, the films are left two minutes after the flame has been removed. The flame should be so adjusted that five to seven minutes are required to melt the crystal.

3. Absolute alcohol is poured over the film, the excess quickly drained off, and the remaining film of alcohol is ignited.

Procedure. Fix the film, preferably by heat. Stain with any good hematoxylin solution, such as Delafield's (or Meyer's hemalum), for 5 to 15 minutes. Wash in tap water for two to five minutes to develop the hematoxylin color. Counterstain either with a 1 : 1000 aqueous eosin solution or with a 0.5 per cent eosin solution in 70 per cent alcohol for 15 to 30 seconds, and then wash and examine.

This method stains nuclei and eosinophilic granules sharply, but it does not stain neutrophilic granules, platelets, malarial parasites, or basophilic cytoplasm in red cells.

Peroxidase Stain: Goodpasture's Stain. This is simple and gives excellent preparations, but some experience with the stain is necessary to get satisfactory results consistently. Formula: sodium nitroprusside, 0.05 Gm.; benzidine (C.P.) Harmer, 0.05 Gm.; basic fuchsin, 0.10 Gm.; alcohol, 95 per cent, 100 ml. The nitroprusside is dissolved in about 1 ml. of water in a mortar. The alcohol and then the other ingredients are added. The stain (without peroxide) should keep for several months. A weak stain is usually due to deterioration of the benzidine, or a poor preparation. It may sometimes be revived by adding a little additional benzidine.

procedure. Use air-dried films, preferably less than 24 hours old. Cover the film with the stain and allow it to stand one minute to fix. Add an equal volume of 1 : 200 dilution of hydrogen peroxide, and stain five minutes. Then wash in water, dry, and mount. It may be counterstained with Wright's stain if desired.

Sato and Sekiya's Stain. This is preferred by some workers. Solutions: (1) Copper sulfate, 0.5 Gm., in 100 ml. water. (2) Benzidine, 0.02 Gm., is ground in a few drops of water, and diluted to 200 ml. and filtered. Then 0.25 ml. of 3 per cent hydrogen peroxide is added. The solution is stored in the dark in a brown bottle. (3) Safranin, 1 per cent aqueous solution.

PROCEDURE. Cover the film with copper sulfate solution for 20 seconds. Drain off and apply the benzidine solution for eight minutes. Drain and stain with safranin solution for two minutes. Wash, dry, and mount.

One must make certain that the solutions used stain the granules in normal blood before attempting to stain pathologic blood.

The granules of the granular leukocytes are stained a deep blue (or brownish in thick overstained films). Myelocytes and premyelocytes contain similar granules. Monocytes contain a smaller number of fine, bluish granules. Lymphocytes never contain granules. The method is, therefore, useful in differentiating lymphocytes from early myelocytes in acute leukemia. The fact that myeloblasts in the strict sense do not show granules, despite earlier statements to the contrary, to some extent restricts the conclusions that can be drawn from the method.

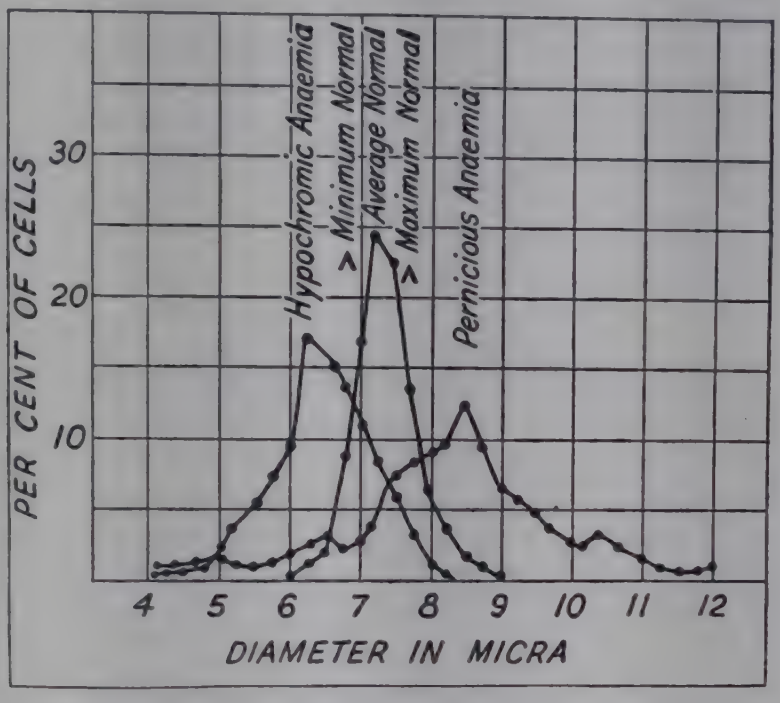
Differential Count. The stained film is inspected, and if the cells are evenly distributed, well stained, and not smudged, it is examined under the oil-immersion lens with a mechanical stage. If the film is on a coverslip, it is desirable to move the slide back and forth from one margin of the film to the other, in the direction in which the films were pulled apart. Every consecutive cell should be identified and recorded until at least 250 cells have been counted. If the count is abnormal a second 250 should be counted on a second coverslip. If the film is made on a slide, the count should be made by moving back and forth at right angles to the margin of the film from the edge of the film to a point several microscopic fields in from the margin. In this way the uneven distribution of leukocytes in such films is partly compensated. As a minimum, 50 cells at four (better five) different parts of the film should be counted (Schilling's four-field-meander method).

Separate headings should be made for each of the types of normal leukocytes and for all the immature types which may be present. The neutrophils should be subdivided into cells with completely segmented nuclei, and cells with incompletely segmented, band-shaped nuclei of mature and immature type. Nucleated red cells should be recorded but not included in the 250 cells counted. Occasional smudged cells must be included, and placed in the proper group if they can be identified, or recorded as smudges if identification is not possible. Films of normal blood containing many smudges are not fit for a count, but in some pathologic conditions in which large, fragile cells are present, it may be impossible to avoid smudges.

Determination of the Diameter of the Red Corpuscles. Price-Jones Curve. By means of a hemacytometer chamber or some accurate stage micrometer, an ocular micrometer is calibrated for use with the oil-immersion lens. It greatly facilitates the measurements if, by altering the length of the draw tube, 50 points on the micrometer scale can be made to coincide with the width of one of the smallest squares of the hemacytometer (50μ). Then measure precisely the diameter of at least 200 red cells (preferably 500) in a thin film stained by Wright's stain. It has been shown that the average diameter in well-made films is slightly less than in fresh preparations. Move the slide by means of a mechanical stage from right to left, with the long axis of the micrometer graduations parallel to that of the slide, and measure to the nearest 0.5μ the horizontal diameter of every round (not distorted) cell passing across the center of the field. Many English observers measure to the nearest 0.25μ, but this is scarcely possible without special projection apparatus. The figures obtained are plotted on a chart, as in accompanying illustration. It is useful also to plot the figures of normal blood for comparison. Such a graph gives a precise estimate of the degree of microcytosis or macrocytosis present and also of the anisocytosis.

Normal blood shows a curve with a high peak and a narrow base. According to Price-Jones the mean figure for the peak in normal blood is 7.2μ, with a possible range from 6.7 to 7.7μ. Many observers in this country have reported figures about 0.4μ higher. Each observer must establish his own normal standard by counts on normal blood.

Two-thirds of the cells fall within 0.5μ of the peak value. In anemias the peak is lower and the base broader. The degree to which the base of the curve is spread out is a measure of the anisocytosis. This may be expressed numerically by calculating a coefficient of variation, which is 5.8 for normal blood, and may reach 13 or more in severe anemias. In pernicious anemia and other macrocytic anemias the peak is at a point above 7.7μ, whereas in most "secondary" anemias, in chlorosis, and in idiopathic hypochromic anemia it is below 6.7μ. The procedure is tedious and impracticable for routine clinical purposes, but in selected cases it gives information that cannot be obtained in any other way.



Price-Jones curves of normal blood, and of cases of hypochromic and pernicious anemia. (Combined and modified from Price-Jones.)

The average diameter of the red cells in a film can be determined simply and quickly, but with somewhat less precision, by means of the *halometer* of Pijper (1929) or the erythrocytometer of Haden. This does not determine the degree of anisocytosis, however.

Thick Films. These are of the greatest practical assistance in searching for malarial parasites (particularly when they are present only in small numbers in the peripheral circulation), for trypanosomes, spirochetes of relapsing fever, and filarial embryos. The method as described by Benavides (1934) has given very satisfactory results at the U. S. Naval Medical School, and is recommended. Experience is necessary in staining and interpreting thick films.

A large drop of blood is spread on a slide with a toothpick, or by tilting the slide, so that the blood is spread over an area ½ inch in diameter. This is *thoroughly* dried in air. It ordinarily requires one to two hours at room temperature, or 30 minutes in the incubator. The film must be protected from insects while drying.

A modified Giemsa stain is used. All utensils must be chemically clean, and the reagents of the highest grade. Two and four-tenths Gm. of Azur II-eosin are placed in an Erlenmeyer flask, 200 ml. anhydrous glycerin are added; the flask is stoppered and heated in a water bath at 60° C. for 30 minutes, during which time it is shaken occasionally. Then 200 ml. anhydrous methyl alcohol are added and mixed; the flask is returned to the water bath and shaken once or twice. It is placed in an incubator overnight and then filtered. This stock stain is stored in tightly stoppered bottles in the dark.

For use, 1 ml. of the stain is added to 30 ml. distilled water (1 or 2 drops to 1 ml.) having a pH of 7.0 to 7.2, or to fresh rain water. The stain and water are mixed and placed in a staining jar. The dried film, without fixation or preliminary laking, is immersed in the diluted stain in a vertical position, and left for an hour and a half. It is rinsed by dipping gently in two changes of water. It is then dried thoroughly, and mounted in oil or balsam. The chromatin of the parasites takes a more brilliant-red color than with the ordinary Giemsa or Wright stain.

Basophilic Aggregation Test (McCord et al., 1924). This is useful in cases of suspected lead poisoning, since it concentrates the cells to some extent and makes the basophilic material more readily visible.

Make films about three times as thick as for a differential count, and dry thoroughly as for thick films. Stain without fixation for 10 minutes with Manson's methylene blue (2 per cent methylene blue and 5 per cent borax in distilled water). Rinse off the stain by dipping the films gently into distilled water several times. Dry thoroughly and examine under the oil-immersion lens. Count the number of clumps of the small dark-blue granules in a large number of fields.

Supravital Staining Methods

Reticulocytes. To demonstrate reticulocytes the red cells must be brought into contact with the stain while fresh and moist. Brilliant cresyl blue is usually employed. We prefer Azur II.

1. Clean coverslips are coated with a thin film of cresyl blue by dipping them into or flooding them with a 0.5 per cent alcoholic solution of the stain, and allowing them to dry. Blood films are made on these coverslips in the usual way and dried in air. They may then be mounted in balsam and examined, or they may be counterstained with Wright's stain. This method is widely used and gives fairly satisfactory results for routine clinical purposes, but it does not show all the reticulocytes.

2. A clean slide is filmed with stain, as described above, and a drop of blood on a clean coverslip is mounted on the slide, rimmed with petroleum jelly, and examined after 30 minutes. This method stains the reticulocytes more heavily and in somewhat larger number. By these methods the count in normal blood is from 0.1 per cent to 0.5 per cent.

3. Osgood's method (1934) is more efficient. In a small test tube, equal volumes of oxalated blood and a 1 per cent solution of cresyl blue in physiological salt solution are mixed and allowed to stand for one minute or more. They are again mixed, and thin films made. These are dried in air, mounted in oil, and examined within 24 hours; or, to secure permanent preparations, they are counterstained with Wright's stain. Blood 24 hours old may be used. This method gives counts two to three times higher than the preceding methods. Osgood's figures for normal blood are 0.5 per cent to 3.8 per cent, averaging 1.5 per cent.

4. One Gm. of Azur II is dissolved in 100 ml. absolute ethyl alcohol. This stock solution seems to work better after standing for about six months.

One part of stock solution is diluted with four parts of absolute ethyl alcohol. Slides cleaned as in method (2) (described on p. 387) are dipped into this working solution of stain, drained, and dried in air. They are polished lightly with lens paper. A drop of blood is touched with the center of a clean coverslip, the coverslip, drop up, is laid on a flat surface, and the slide lowered gently onto it so that the weight of the slide causes even spreading of the drop. The coverslip is sealed with petroleum jelly and examination made after 10 minutes. This method stains the reticulocytes with maximum intensity, so that even single granulations stand out conspicuously. Counts obtained by this method are much higher than by the usual methods and cannot be compared directly with them. The leukocytes are also stained by this procedure, and retain their vitality and motility for a considerable time. The lymphocytes are recognized by a single nucleolus; neutrophils, by sharp, elongated, finger-like pseudopods; eosinophils, by a regular, mosaic

type of granulation; basophils, by irregular clumping of the granules and early staining of the nucleus; and monocytes, by blunt, wide pseudopods and occasionally by the ingestion of foreign particles.

This method has been used with satisfaction in the laboratories of the U. S. Naval Medical School.

On a film stained by any of these methods the total number of red cells and of reticulocytes in a series of oil-immersion fields is counted until 1000 red cells have been counted. To reduce the size of the field to practicable dimensions a piece of stiff paper with a square hole about ⅛ inch in diameter cut in the center is inserted in the eye piece.

Leukocytes (Sabin's Method). 1. Perfectly clean slides are brushed free from dust, flamed, and dipped in a dilute alcoholic solution of biologic neutral red. This is drained off immediately, and the slides are placed upright on one end and dried in air. For average blood a 0.04 per cent solution is used. A stock solution is prepared by dissolving 0.1 Gm. of neutral red in 10 ml. absolute alcohol. For use, 0.4 ml. of this solution is added to 10 ml. absolute alcohol.

2. To 2 ml. of this dilute solution are added 3 drops of a saturated solution of Janus green in absolute alcohol. Similarly slides are prepared with this solution of the combined stains.

With a clean coverslip a drop of fresh blood is mounted on one of these slides as in making an ordinary fresh preparation. It must spread evenly without pressure. The coverslip is rimmed with petrolatum, and the slide placed at once in a warm box or on a thermostatically controlled warm stage, at 37° C. It is examined with the oil-immersion lens, care being taken not to jam the objective down onto the coverslip when focusing.

For most purposes the neutral red alone is preferable, as it is practically nontoxic, and the cells usually remain alive and motile for an hour or more. Janus green stains the mitochondria which are numerous in immature cells, and thus gives additional information, but it is quite toxic. The cells usually lose their motility and die after 15 to 20 minutes.

In neutral-red preparations the granules of the *polymorphonuclear neutrophils* appear as uniform, fine, faintly reddish-stained structures. There may be one or two red vacuoles present. The healthy cells show constant active ameboid motility with streaming of the granules within the cell. They often move across the field with surprising rapidity. Except when dying they are rarely seen rounded up, as in fixed films.

The *eosinophils* contain round, coarse granules of uniform size, somewhat more deeply stained than the neutrophilic granules. They also show motility, but are somewhat less active than the neutrophils.

The *basophils* show round granules varying in size and depth of staining, but some are usually more deeply stained than in the preceding types. They show sluggish ameboid motility.

The granules of the *myelocytes* take the red stain in a similar manner. These cells are not motile (except in hanging drop), show no streaming of the granules, and contain no vacuoles.

The *lymphocytes* show a strikingly clear cytoplasm without any granules. They contain from one to several reddish-staining vacuoles which are variable in size and shape, and may increase in size as the preparation stands. With Janus green they typically show a clump of relatively coarse, rod-shaped mitochondria opposite the nucleus. The large lymphocytes and many of the small ones show little or no motility, but many of the intermediate forms show sluggish progressive ameboid motility which is characterized by the fact that the nucleus is usually in the front of the advancing cell, and the posterior end is prolonged into a blunt tail-like projection giving the cell the shape of a hand mirror. The coarse chromatin masses in the nucleus can easily be seen.

The *monocytes* show red particles which vary from fine granulations to coarse vacuoles. They usually contain moderately numerous, small vacuoles, coarser and redder than the

neutrophilic granules. In the moving cell these show streaming within the cytoplasm, but when the cell rounds up they tend to be arranged in a rosette pattern about the centrosphere in the concavity of the nucleus. The cells often show progressive ameboid motility which is much more sluggish than that of neutrophils and of a "sliding" type. They may round up, and show a nearly constant wavy motion of their margins, with numerous, relatively small pseudopods which may be slender and filiform, or broad and ruffle-shaped, quite similar to the irregularities occasionally seen in stained films. They are actively phagocytic. Janus green stains fine mitochondria diffusely scattered through the cytoplasm.

Sabin differentiates the monocytes sharply from the *clasmatocytes,* or *histiocytes,* the wandering phagocytic cells of the reticulum, which appear in the blood in certain pathologic conditions, and occasionally in small numbers in normal blood. These cells are more actively motile and phagocytic than the monocyte. They show more numerous vacuoles which are often coarser and vary more in size and color than in the case of the monocyte, and show no tendency to assume the rosette pattern.

In the hands of Sabin and her associates this method of staining has been of great value as a research procedure. For ordinary clinical purposes its chief value is in the identification of monocytes, and in their differentiation from myelocytes on the one hand and from lymphocytes on the other. The distinguishing criteria are more definite and decisive than in the case of stained films. It is also of assistance in the recognition of immature cells, rich in mitochondria.

Myeloblasts contain many mitochondria diffusely scattered throughout the cytoplasm. They are minute, spherical, comma-shaped or fine, threadlike bodies. They persist in the earlier myelocyte stages, but may be hidden by specific granules. In the lymphocytes the mitochondria are coarser, short, thick rods which may be arranged in one or two rows encircling the nucleus, or may be clumped opposite the indentation of the nucleus. In primitive monocytes they appear as numerous, fine, spherical or slender rodlike bodies, diminishing in number as the cell matures.

Sabin emphasizes that no valid conclusions can be drawn from preparations in which the cells are dead. She regards any staining of the nucleus as indicating a dead or dying cell. Those not skilled in the procedure are advised to depend mainly upon preparations stained with neutral red alone.

Miscellaneous Examination

Fragility of the Red Corpuscles (*Resistance of Red Cells to Hypotonic Salt Solution*). 1. In a porcelain evaporating dish put a few grams of pure sodium chloride, and heat over a flame with constant stirring until all moisture has been driven off. Cool in a desiccator. Make a 1 per cent solution by dissolving exactly 1 Gm. in distilled water in a 100-ml. volumetric flask, dilute to volume and mix.

2. In test-tube racks, two series of 15 small test tubes each are set up, using glass which has been acid-cleaned, thoroughly rinsed (the last time in distilled water), and dried. In each set of tubes is prepared a series of dilutions of salt solution, ranging from 0.3 per cent to 0.6 per cent, in steps of 0.02 per cent, with a volume of 2 ml. in each tube. This may be done conveniently by adding to each tube in each set (with a serologic pipet) first 1 per cent salt solution beginning with 0.6 ml. in the first tube, and increasing the quantity by 0.04 ml. in each succeeding tube; and, second, distilled water, beginning with 1.40 ml. in the first tube, and diminishing the quantity by 0.04 ml. in each successive tube. In laboratories in which many tests are made it is simpler to prepare each dilution in 100 ml. quantities, and add 2 ml. of the proper dilution to the corresponding tubes when setting up the test. In tightly stoppered bottles they keep for several months.

3. To each tube in the first set one drop of the blood to be examined is added, and to each tube in the second set one drop of normal (control) blood. The control series with normal blood must not be omitted. One may aspirate 2 ml. of blood from a vein and

drop it directly into the tubes from the tip of the needle, without any anticoagulant. This necessitates precise and speedy manipulation to avoid clotting. We prefer to aspirate the blood into a syringe containing one-tenth its volume of 3 per cent sodium citrate to prevent clotting (oxalate may be used). Pack the cells in a graduated centrifuge tube, pipet off the supernatant fluid, and suspend the cells in an equal volume of physiological salt solution. With a capillary pipet add one drop of cell suspension to each tube. The syringe (as well as the glassware) used must be dry or rinsed with physiological salt solution.

4. The tubes are shaken well and set aside (at room temperature or in the icebox) for two hours or more, until sedimentation is complete.

5. Record: (1) the concentration of salt in the first tube in each series in which a trace of hemolysis appears (the minimal resistance, normally about 0.44 per cent); and (2) that in the first tube in each series in which hemolysis is complete and there is no red sediment (normally about 0.32 per cent). The presence of a little colorless sediment (stroma) is disregarded.

6. Compare the figures for the blood in question with those for the normal control. A clear-cut difference of 0.04 per cent may be regarded as significant.

The resistance is diminished (fragility increased) chiefly in hemolytic jaundice. In most other types of anemia and in obstructive jaundice the resistance is normal or slightly increased. It is much increased in Mediterranean anemia, in which the cells are thin.

Gänsslen (1922) and Haden (1934) have shown that the diminished resistance is probably not due to any abnormality in the chemical composition of the cell, but to its unusual shape. When red cells are immersed in hypotonic salt solution they absorb water and swell until the osmotic pressure is equalized. The normal red cell, which is a flat disc, can increase markedly in volume by assuming a more spherical shape, before physical rupture of the cell occurs. The cells in hemolytic jaundice are narrower and thicker, are more nearly spherical to start with, and a relatively slight degree of swelling suffices to cause their rupture and hemolysis. There is no constant relation between diminished resistance to hypotonic salt solution and susceptibility to other hemolytic agents.

Sedimentation Rate of the Red Corpuscles. This is determined by filling a suitable graduated tube with well-mixed oxalated blood, and either observing the time required for the upper level of the column of red cells to fall to some fixed point in the tube, or, preferably, observing the amount of fall during certain definite time intervals. Many different methods, each with its own special tubes, have been employed, of which those of Linzenmeyer, Westergren, and Cutler are the best known. These give equivalent results, but the normal figures are different for each method. The results would be comparable if expressed as the percentage of the length of the column (as in Wintrobe's tubes) instead of in millimeters.

Wintrobe and Landsburg's Method. We have found Wintrobe's hematocrit tube satisfactory and very convenient, since with it an accurate determination of the cell volume can subsequently be made with the same specimen of blood. The tube is filled to the 10-cm. mark with well-mixed oxalated blood (see section on Determination of Cell Volume). Blood may be used within 2 or 3 hours after venepuncture; older specimens give too slow a rate. The tube is placed in *exactly* a vertical position in a suitable rack, or in plasticine. It must not be moved or jarred during the period of observation. Reading is made at the end of one hour, and in special cases also after 10, 20, and 30 minutes. The cell volume is determined as previously directed. A correction (approximate) for the accelerating effect of anemia, if present, may be made by means of the chart, p. 396, from Whitby and Hynes (1938).

The corrected rate for normal individuals during the first hour is 2 to 9 mm. (rarely over 6 mm.) for males and also for females, if the same figure (45) for normal cell volume is used, although there is greater variation among females than among males. Although this correction largely eliminates the effect of dilution on the sedimentation rate

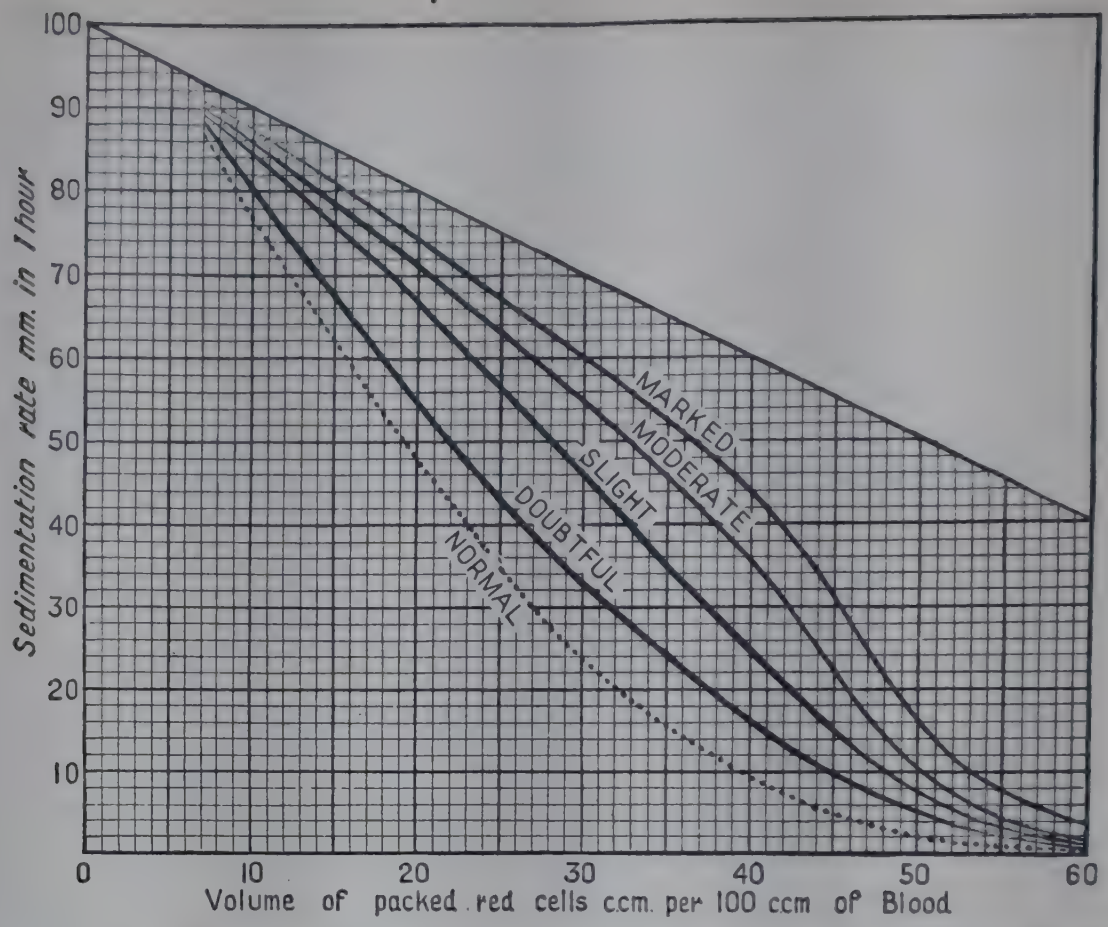
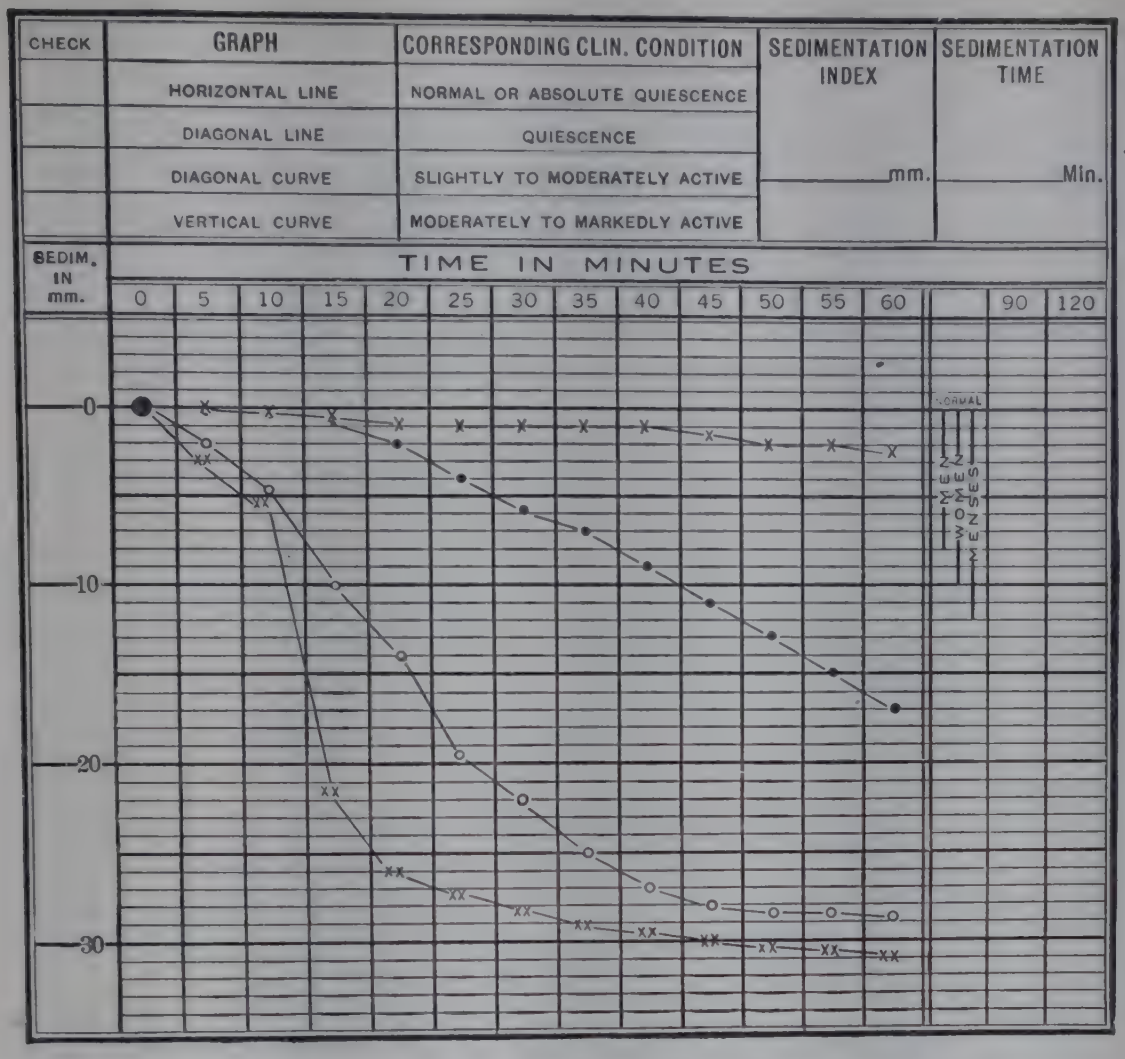


Chart for correction of sedimentation rate for anemia by means of the corpuscular volume. To correct for anemia, find the junction of the lines of the observed sedimentation rate and the observed corpuscular volume; this point will fall in one of the five zones (normal, doubtful, etc.) which indicate the approximate degree of increase in the rate. If a definite compensated figure is required, follow the appropriate curve down to the point where it cuts the 45 ccm. thick vertical line. Example: Observed rate = 68 mm.; observed corpuscular volume = 20 ccm.; point of junction lies in area of "slight" increase; compensated rate = 16 mm. (From Whitby and Britton: Disorders of the Blood, Philadelphia, The Blakiston Company, after Whitby and Hynes.)

in normal blood, it appears to add little to the precision of the test when applied to pathologic cases. In patients with severe anemia the charts tend to give an excessive correction. The uncorrected as well as the corrected figures, therefore, should be recorded in all cases.

Cutler's Method. This is simple and has been extensively employed. Into a dry, sterile syringe aspirate 0.5 ml. of sterile 3 per cent sodium citrate solution. Puncture the vein, and aspirate blood to the 5-ml. mark. Draw back the piston, mix the contents of the syringe, remove the needle, and expel the blood into a Cutler sedimentation tube. Place the tube in a rack in a vertical position, and record the upper level of the column of red cells every five minutes for an hour. The observations may be plotted on special charts. The normal readings at one hour are given as 2 to 8 mm. for men and 2 to 10 mm. for women.

Significance. Many different factors influence the sedimentation rate, and the mechanism of the process is not understood. In general, the higher the column of blood, the greater will be the fall (in mm.) in a given interval. The rate varies inversely with the number of red cells present. It is markedly accelerated in a variety of pathologic conditions in which there is an increase in the globulin, and



Cutler's graphic chart for recording blood sedimentation. (*American Journal of the Medical Sciences,* June 1926.)

———×———×———Horizontal line (clinically healthy individual).
———•———•———Diagonal line (clinically quiescent tuberculosis).
———○———○———Diagonal curve (clinically slightly active tuberculosis).
———××———××———Vertical curve (clinically markedly active tuberculosis).

particularly in the fibrinogen of the plasma. Microscopic examination shows that the accelerated sedimentation rate is associated with, and closely parallels, increased rouleaux formation (Fahraeus). The rouleaux are much larger and more compact than in normal blood.

An accelerated sedimentation rate has been observed empirically chiefly in: (1) Anemias of all types. (2) Normal pregnancy after the second or third month, and the puerperium. (3) Acute infections of all types, and active chronic infections, including syphilis and tuberculosis. (4) "Nephrosis." (5) Advanced malignant tumors. (6) Internal hemorrhages. (7) Any condition associated with necrosis or extensive destruction of tissue, including coronary thrombosis. It is not, as a rule, increased in relatively inactive, chronic focal infections, in benign tumors, in unruptured ectopic pregnancy, nor in purely psychoneurotic states.

It is thus not specific, and like fever or leukocytosis, it is not diagnostic of any particular disease. It is occasionally not accelerated in individuals showing pathologic conditions in which this would be expected. An accelerated rate points to the existence of significant organic disease, and indicates the need for thorough in-

vestigation. We have found it useful, on this basis, as part of a routine diagnostic study.

In general there is a rough parallelism between the activity of an infection and the degree of acceleration of the rate, particularly the fall during the first 15 to 30 minutes. The relationship in the case of tuberculosis is shown in the preceding chart from Cutler. He regards the sedimentation rate as more valuable than the temperature curve, the pulse rate, or the body weight as an aid in estimating the activity of tuberculosis and in guiding treatment and advocates the test as a routine procedure in the study of this disease.

Blood Volume. This may be determined by the method of Keith, Rountree, and Geraghty.

1. Prepare a 1.5 per cent solution of Congo red (biologically tested) by dissolving 0.375 Gm. in 25 ml. freshly distilled water; filter, sterilize by boiling, and cool. (If the dye preparation is not known to be suitable, test for toxicity by injecting some of the solution intravenously into a dog.)
2. Withdraw just 10 ml. of blood with a sterile, dry, calibrated record syringe and needle, expel into a graduated 15-ml. centrifuge tube containing 2 ml. of 1.1 per cent sodium oxalate solution, and mix.
3. Inject the dye solution intravenously through the same needle, avoiding leakage. The dose in ml. is determined by dividing the body weight in kilograms by 4.
4. Four minutes later (not less than three nor more than six), withdraw 10 ml. blood from the other arm with a different clean needle and syringe, and put into a second similar centrifuge tube containing 2 ml. of the oxalate solution.
5. Centrifuge both tubes until the cells are packed. Record the total volume and the volume of packed cells in each tube, and take the average. The volume of diluted plasma is determined by subtraction.
6. Prepare a standard by adding to 2 ml. of diluted plasma from the first tube, 4 ml. of 0.9 per cent sodium chloride solution, and 2 ml. of a 1 : 200 dilution of the 1.5 per cent solution of the dye.
7. To 2 ml. of diluted plasma from the second tube add 6 ml. of 0.9 per cent sodium chloride solution.
8. Read in colorimeter.
9. The dilution factor is determined as follows. The plasma in both tubes has been diluted with 2 ml. of oxalate solution. The actual plasma volume is therefore ml. of diluted plasma — 2 ml.

$$\text{Dilution factor} = \frac{\text{Volume of diluted plasma} - 2}{\text{Volume of diluted plasma}} \text{ (usually about 0.75).}$$

Plasma per cent = actual plasma volume / 10.

Calculation: If a Duboscq type of colorimeter is used:

$$\text{Total blood plasma in ml.} = \frac{\text{Reading of standard}}{\text{Reading of unknown}} \times \text{ml. of dye solution injected} \times 200 \times \text{dilution factor.}$$

$$\text{Total blood volume in ml.} = \frac{\text{Total plasma volume} \times 100}{\text{Plasma per cent}}$$

In this procedure hemolysis must be scrupulously avoided or gross errors will be introduced.

The result is usually expressed in terms of ml. of blood per kilogram of body weight. The usual normal for adults is 70 to 90 ml. per kilogram. The method is not applicable to patients with "nephrosis" or amyloidosis. (See Congo red test for amyloidosis.)

By the *carbon monoxide method* the average normal volume is 60 to 70 ml. per kilogram. It is preferable in conditions showing abnormalities in the red cell count, but requires the special equipment and training necessary for precise gas analyses. The reason for the difference in these figures is that the carbon monoxide is all combined with hemoglobin within the cells, whereas the dye, which is dissolved in the plasma, penetrates into partly collapsed capillaries into which the cells cannot enter. Both methods measure only the blood which is in active circulation and not that which is in the spleen pulp and other stagnant areas.

The blood volume shows remarkably little variation in normal individuals in spite of great irregularities in fluid intake and excretion. It is markedly increased in erythremia. Increases (usually less marked) have been reported in the secondary polycythemias, and slight increases in leukemia, in conditions associated with splenomegaly, and particularly during the latter half of pregnancy. Marked decrease occurs in shock following acute hemorrhage and in conditions associated with dehydration. The volume is normal in essential hypertension and chronic nephritis.

Biopsy Examinations. These examinations frequently give information of great diagnostic value. Sections of enlarged lymph nodes, fixed and stained by the usual methods, usually give an immediate, definite diagnosis in cases of Hodgkin's disease, lymphosarcoma, or lymphatic leukemia, either in the leukemic or leukopenic stage. Examination of the *bone marrow* may be equally helpful. A small portion can be removed with a trephine under local anesthesia, usually from the sternum. The tibia has not proved to be a satisfactory site for biopsy, since the tibial marrow often fails to show hyperplasia when it is present in the proximal long bones. Portions of tissue obtained by trephining should be fixed in formalin, Zenker, or sublimate-alcohol, and cut in thin sections. If stained with Wright's stain, the specific granulations of the leukocytes can be made out. One may also make imprint preparations or thin smears of the marrow on clean coverslips. A better method (Isaacs) is to make a fairly thick suspension of marrow cells in homologous plasma or serum (it must be from the same blood group), by teasing and gently agitating a bit of marrow in a few drops of serum. Films from such a suspension are made and stained in the same way as ordinary blood films. In satisfactory preparations the fine details of cell structure are preserved, and accurate differential counts can be made.

In patients with serious blood diseases such procedures are not without risk, both of infection and bleeding. They should be performed with asepsis as strict as that demanded for a laparotomy.

Sternal Marrow Aspiration. A study of sternal marrow obtained by aspiration by a modification of Arinkin's method has yielded results of great practical value and should be a routine procedure in most cases of blood dyscrasia in which the diagnosis is in doubt. Although not so dependable as a biopsy, this obviates the need of the latter in many cases. If the results are inconclusive, however, and particularly if they suggest an acellular or "aplastic" marrow, a biopsy should also be carried out.

A No. 18 gauge lumbar-puncture needle 4 cm. long is used (aseptic precautions, procaine infiltration). With the point at the center of the sternomanubrial junction, the needle is held at an angle of 60 degrees with the chest wall, pointing caudad, and gently forced through the external lamina. Care must be taken not to let the needle jump too deeply into the sternum when the lamina gives way. The base of the needle is then depressed to an angle of 30 degrees, and the needle is gently forced in to a depth of 1 to 1.5 cm. The stylet is removed, a 2 ml. syringe is attached, and 0.2 ml. (not over 0.5 ml.) of marrow contents is gently aspirated. Removal of a larger volume is apt to result in an undesirable dilution of the marrow contents with blood. If necessary the needle is rotated, and the position of the tip varied until material is obtained. The material, which looks like blood, is expelled into a small tube containing 2 mg. potassium oxalate per ml. The needle is removed, and the puncture wound sealed with collodion.

Films and cell counts are made from this just as from oxalated blood. The cell characteristics are clearly defined.

The procedure is practically painless, and without risk provided asepsis is perfect and the needle is not pushed entirely through the sternum so that the possibility of injury to mediastinal structures or introduction of infection is avoided. The needle should be provided with a movable guard to prevent this accident. Some prefer to drive the needle through the lamina by tapping gently with a small mallet.

A thorough acquaintance with normal marrow is essential if valid conclusions are to be drawn regarding pathologic changes. Total nucleated cell counts vary widely, from 10,000 to 200,000 per cu. mm., depending largely upon the degree to which marrow contents are diluted with circulating blood. They are therefore of little value, except that a high count indicates success in obtaining relatively undiluted marrow. The usual range of differential cell counts in normal marrow is given in Table 43. Repeated differential counts of the same specimen of marrow vary much more than in the case of films from the circulating blood, because of irregularity in the distribution of the cells and because there are more cells which are difficult to identify. Slight variations from the usual range must be interpreted with caution.

Examination of the marrow has proved valuable in determining the type and extent of hyperplasia present, both in the anemias and the leukemias. It is especially useful in the differentiation of simple leukopenia, aplastic anemia and leukopenic leukemia. The characteristic cells of Gaucher's disease and multiple myeloma can usually be recognized. In malaria there is often a larger proportion of infected erythrocytes than in the peripheral blood. It is a safer procedure than splenic puncture for the demonstration of Leishmania.

Table 43

DIFFERENTIAL COUNT OF NUCLEATED CELLS IN FILMS OF NORMAL BONE MARROW

	Compiled		*Whitby and Britton Range*
	Range	*Average*	
Myeloblasts	0.5 – 5	2.0	0 – 2.5
Promyelocytes (undifferentiated)	1 – 8	5.0	0.5– 5.0
Neutrophils			
Myelocytes	5 –20	12.0	2 – 8
Metamyelocytes ("juveniles")	10 –20	15.0	2.5–12
Nonfilament ("Stabzellen")	5 –15	10.0	
Segmented	15 –30	20.0	20 –50
Eosinophils			
Myelocytes	0.5 – 3	1.5	0 – 1
Segmented	0.5 – 4	2.0	0 – 4
Basophils			
Myelocytes	0 – 0.5	0.3	
Segmented	0 – 0.7	0.2	0 – 1
Lymphocytes	5 –20	10.0	5 –20
Plasma cells	0 – 2	0.4	0 – 1
Monocytes	0.5 – 5	2.0	0 – 5
Reticulum cells	0.2 – 2	0.2	
Megakaryocytes	0.03– 3	0.5	
Megaloblasts			0 – 4
Primitive erythroblasts (macroblasts)	0.5 – 4	1.5	
Immature normoblasts ("erythroblasts")	1 – 5	2.0	2 – 7
Normoblasts	10 –30	20.0	7 –19
Ratio leukocytes to nucleated red cells	2 : 1– 8 : 1	4 : 1	2 : 1 – 8 : 1

Note: In infants and young children there is usually a somewhat higher percentage of immature forms, both in the leukocyte and erythrocyte series.

Coagulation Factors

Coagulation Time and Retractility of the Clot (Syneresis): Coagulation Time (Lee and White). (1) In a rack set up four small test tubes with an internal diameter of 8 mm. They must be chemically clean, and must be rinsed with physiological salt solution just before the test. (2) A sterile 5-ml. (or larger) syringe and needle are rinsed out with sterile physiological salt solution, and the last of the salt solution is expelled with the needle held vertically, so that the needle and the dead space in the tip of the syringe are left filled. (3) Withdraw 5 to 7 ml. of blood from a vein. The validity of the test depends upon entering the vein cleanly at the first attempt. Suction must be gentle to avoid drawing air bubbles into the blood. (4) Remove the needle and expel 1 ml. of blood gently into each of the first three tubes. In the fourth tube place 2 or 3 ml. if available. Place the first three tubes in a thermostat or water bath at 37° C. The fourth tube is used in the determination of retractility of the clot (see below). (5) After five minutes (or less, if a shortened coagulation time is suspected) remove the first tube from the rack, and tilt gently to determine whether coagulation has occurred. (6) After one-half minute examine the second tube in the same way. (7) After one-half minute more again examine the first tube, and continue this procedure alternately at one-half-minute intervals, until coagulation has progressed so far that the tube can be inverted without spilling the blood. (8) Then the third tube, not yet disturbed, is examined. If coagulation is not complete, re-examine the tube at one-half-minute intervals until it can be inverted. The total interval from the time the blood first enters the syringe until this tube can be inverted measures the coagulation time.

Normal blood coagulates in from 6 to 12 (or even 15) minutes; more often 12 than 6, if the technic has been perfect. Practically all technical errors, except a low temperature, hasten coagulation. The coagulation time is longer if tubes of larger diameter are used.

Retractility of the Clot. The fourth tube is put in an incubator and is inspected after one hour, and again after 18 to 24 hours. Normally retraction of the clot and expression of serum is appreciable after one hour and is marked at the later period. Delayed and incomplete retraction is met with in blood which is deficient in platelets. Occasionally, however, a clot of normal blood fails to separate from the walls of the tube, but if such a clot is loosened with a platinum wire, retraction occurs promptly.

Coagulation Time with Capillary Blood. This is less accurate because of the admixture with the blood of undeterminable amounts of tissue juice. The finger tip is cleansed in the usual way and a deep puncture made, to insure a free flow of blood. The first drop is wiped off, and from the second drop one or more clean capillary tubes about 1.5 mm. in diameter are filled (by capillary attraction). (The tubes should be kept as nearly at body temperature as possible.) After two minutes, at one-half-minute intervals, a scratch is made with a file on the capillary tube about 1 cm. from the end; the 1-cm. portion of the tube is snapped off gently and the broken ends separated. Coagulation is regarded as present when threads of fibrin can be seen bridging the gap of a few millimeters between the ends. The time which has elapsed since the appearance of the drop of blood on the skin is the coagulation time, normally about three to six minutes.

Bleeding Time (Duke). Make a deep prick in the lobe of the ear or in the finger. At half-minute intervals remove the drop of blood with a piece of filter paper, taking care not to touch the skin. Note the time that elapses before bleeding ceases. Normally this does not exceed three minutes. The bleeding time is prolonged in thrombocytopenia and in conditions associated with reduction in fibrinogen. It is usually normal in hemophilia.

Prothrombin Time (Quick). (1) In a tube 100 × 12 mm. put 0.2 ml. M/10 sodium oxalate solution (or 0.05 ml. in a Kahn tube, 75 × 10 mm. if capillary blood must be used as in infants). (2) Add blood fresh from the vein up to the 2-ml. mark (or to the 0.5-ml. mark if capillary blood is used), mixing thoroughly. (3) Centrifugalize at low

speed until the cells have been thrown down. (4) In a Kahn tube put 0.1 ml. plasma and 0.1 ml. of thromboplastin solution, and put in water bath at 37° C. (5) Add 0.1 ml. warm M/40 calcium chloride solution, and start the stop watch. (6) Replace tube in water bath, removing momentarily at frequent intervals and tilting slightly, until clotting occurs. (7) Immediately record the time with the stop watch. (8) Repeat the entire process, using normal plasma.

The result is usually expressed in terms of the percentage of normal.

$$\text{Clotting activity} = \frac{\text{Clotting time of normal plasma}}{\text{Clotting time of patient's plasma}} \times 100$$

Bedside method of Smith, et al. (1) In a Kahn tube put 0.1 ml. thromboplastin solution. (2) Add 0.9 ml. blood *freshly* drawn from the patient, invert once to mix, and immediately record time with stop watch. (3) At intervals of 1 to 2 seconds tilt gently, until clotting occurs. (4) Record time with stop watch. (5) Repeat using normal blood. Both must be carried out at the same temperature. Calculate as directed above.

This method is less precise than the preceding, but suffices for practical purposes.

The relationship of the *quantity* of prothrombin to the prothrombin *time* is not linear, but is expressed by a curve. As the amount of prothrombin is progressively diminished, there is but little prolongation of the time until the quantity is reduced to below 50 per cent of normal. Further reductions in quantity cause progressively greater increases in the prothrombin time. To detect lesser degrees of reduction of prothrombin and measure them with any precision or to detect a possible increase in activity, it is necessary to use plasma which has been diluted with physiological salt solution (1 in 3 to 1 in 8). The clinical significance of such slight changes has not been well established.

Solutions. M/10 sodium oxalate. Dissolve 1.34 Gm. anhydrous sodium oxalate in 100 ml. of distilled water.

M/40 calcium chloride. Dissolve 1.11 Gm. anhydrous calcium chloride in 400 ml. distilled water.

Thromboplastin. (1) Thoroughly grind up beef or rabbit lung. (2) To each 10 Gm. of finely ground lung add 10 ml. of physiological salt solution. (3) Stir frequently for two to three hours. (4) Strain through gauze. (5) Add merthiolate to make a dilution of 1 : 1000 and store in the icebox. Such preparations at the U. S. Naval Medical School have remained usable for three to five months.

It is desirable to adjust the activity of the solution, by dilution if necessary, so that normal plasma will clot in from 25 to 60 seconds. If a longer time is required, the preparation should be discarded.

Dried rabbit brain may also be used. Commercial preparations are available and are desirable for small laboratories in which relatively few tests are made.

Capillary Resistance Test (Rumpel-Leede Phenomenon). Apply a blood-pressure cuff about the upper arm and keep inflated for five minutes at or slightly above the diastolic pressure. If reaction to the test is positive, within a few minutes after the cuff is removed, a crop of purpuric spots will appear in the skin below the cuff. A few small spots are of no significance, but a profuse crop with spots of large size (over 1 cm. in diameter) is almost pathognomonic of thrombocytopenic purpura.

Fibrinogen Content. A method for the quantitative estimation of fibrinogen, in conjunction with other plasma proteins, is given on p. 787.

Tests for Isoagglutination

Human Blood Groups

When the serum of certain individuals is mixed with a suspension of red cells from certain other individuals, agglutination of the cells occurs. By a study of these

reactions Landsteiner (1900) was able to divide human beings into three distinct groups. The work of his associates, Decastello and Sturli (1902), indicated the existence of a fourth group, which was described as such by Landsteiner (1909). This last publication escaped general notice, and Jansky (1907) and Moss (1910) independently demonstrated the existence of the same four groups, although they gave them different numerical designations. Moss's work was of great practical value because he described a simple method for group determinations which could be used clinically, and because he brought to general notice the practical importance of the procedure in the selection of suitable donors for transfusion. These discoveries removed the major peril in transfusions and made their extensive use possible for the first time.

The *interrelationships between the cells and sera of the four groups* can be most readily understood by inspection of Table 44.

It is evident, first, that red cells are not agglutinated by the serum of another member of the same group. Serum from Group AB (Group 1 of Moss) has no agglutinating activity, whereas cells of this group are agglutinated by serum of the other three groups. Cells of Group O (Group 4 of Moss) are inagglutinable, whereas the serum agglutinates cells of the other three groups. Blood from Group A (2) and from Group B (3) cross agglutinate each other's cells, and both also agglutinate the cells of Group AB, while the cells of both are agglutinated by Group O serum.

Table 44

TABLE TO SHOW THE RELATIONSHIPS OF THE CELLS AND SERUM OF THE DIFFERENT BLOOD GROUPS

International Group	*Cells AB*	*Cells A*	*Cells B*	*Cells O*	*Cell Receptors*	*Serum Agglutinins*	*Moss Group*	*Jansky Group*	*Frequency Per Cent*	*May Give Blood to*	*May Receive Blood from*
Serum Group AB	0	0	0	0	AB	0	1	4	6	AB	All
Serum Group A	+	0	+	0	A	b	2	2	40	A AB	A O
Serum Group B	+	+	0	0	B	a	3	3	10	B AB	B O
Serum Group O	+	+	+	0	0	ab	4	1	44	All	O

Agglutination is indicated by +, and the absence of agglutination by 0.

These observed facts can be explained by means of the following *hypothesis*. In human serum there may occur *two different isoagglutinins*, designated arbitrarily *a*, and *b*. In human red cells there may occur *two corresponding agglutinogens, or receptors*, A and B. Any given serum may contain either or both of these agglutinins, or neither of them. Similarly, in a given cell, either or both of the receptors may be present, or neither of them. However, an agglutinin and its corresponding receptor are never both present in the same blood, but one or both of the agglutinins are practically always present if the corresponding receptors are absent. In a mixture of bloods, agglutination will occur

whenever an agglutinin and its corresponding receptor are present. The distribution of agglutinins and receptors which is believed to occur is also shown in Table 44. The correctness of this hypothesis is supported by agglutinin absorption tests.

The characteristics of the cells (*possession of receptors*) is the factor which determines the group. This is a *constitutional trait* which is inherited as a dominant Mendelian character. The group characters are often incompletely developed at birth, especially the agglutinins, but become evident after 3 to 12 months. After these have once developed, the *group never changes,* although the agglutinating activity of the serum may vary or even disappear. Since the possession of receptors by the red cells is the factor which determines the blood group, it is preferable to designate the groups by symbols which indicate their receptor content, rather than by arbitrarily chosen numerals. The four groups are therefore termed A, B, AB, and O (no receptors).

Table 44 shows the approximate *frequency of the groups* in the Caucasian races of Europe and North America. In some of the races of Asia and Africa Groups B and AB are relatively more frequent, whereas in American Indians Group O is much more frequent. All four groups occur in all the human races which have been studied and, according to Landsteiner, also in the orang.

In about one-fifth of the cases a serum which shows agglutinating activity will also hemolyze the red cells, if it is fresh and active. *Hemolysis* never occurs in the absence of agglutinins, although the process of agglutination may be masked by hemolysis. Inactivation prevents hemolysis, but does not affect agglutination.

The *practical importance* of blood group determinations depends largely upon its use in *selecting suitable donors for transfusion.* It has been found that blood is suitable for transfusion if the cells of the donor are not affected by the serum of the recipient. On the other hand, if the donor's cells are agglutinated (or hemolyzed) *in vitro* by the recipient's serum, these cells will be destroyed in the body of the recipient, and will usually cause a violent and sometimes fatal reaction, often with hemoglobinuria and jaundice. Although it seems preferable theoretically that the donor and recipient should belong to the same group, in practice it is usually immaterial if the cells of the recipient are agglutinated by the serum of the donor. Unless an unusually large transfusion is given, or the agglutinin titer of the donor's serum is unusually high, the activity of the serum of the donor is so modified in the body of the recipient, by dilution or otherwise, that its agglutinative and hemolytic properties are abolished. Such transfusions do not cause minor reactions more frequently than those in which a donor of the same group is used. With important limitations to be discussed (p. 407), a Group O individual can be regarded as a *universal donor,* since the cells are not agglutinable, and a Group AB individual may be regarded as a *universal recipient.*

Selection of a Donor. The donor must be a healthy individual, preferably a young man, free from infectious disease. Syphilis must be excluded with particular care, as the blood in the primary and secondary stages is highly infectious. A Wassermann reaction or a flocculation test should always be done, but a history and complete physical examination are also indispensable. We have seen syphilis conveyed by blood from a donor with primary syphilis, but a negative Wassermann reaction. The risk is probably small in late

tertiary or latent cases. Malaria has been transmitted in a number of instances, even from latent cases, and no one with a history of recent infection should be used as a donor. Other infections the transmission of which has been reported include measles, smallpox, influenza, typhoid fever, infectious hepatitis, and tuberculosis.

The blood group of both donor and recipient should be determined. If this indicates that the blood is compatible, a check is made by testing the action of the recipient's serum on the donor's cells. If a universal donor is to be used for the first time for a recipient of a different group, the agglutinin titer of the donor's serum for the recipient's cells is determined (see p. 407).

Group Determination: PROCEDURE. There are required: (1) Serum known to have high agglutinating activity from Group A, Group B, and preferably also Group O. (2) Serum from the patient and each prospective donor. (3) A cell suspension from each, prepared by adding one or two drops of blood to 5 ml. of a 1.5 per cent sodium citrate solution in physiological salt solution.

The following preparations are set up with cells from each of the individuals to be tested:

1. Unknown cells + Group A serum.
2. Unknown cells + Group B serum.
3. Unknown cells + Group O serum.

The third preparation is not essential, but it serves as a valuable check on the accuracy of the results of the first two preparations.

On a slide, mix one drop of serum and one drop of cell suspension. A small drop of the mixture is transferred to the center of a 22-mm. coverslip, which is then inverted over the concavity of a hollow ground slide and sealed with petrolatum. The drop is inspected at once under the low power. The number of red cells should be sufficient to form a single layer, after they have settled to the surface of the drop, with the cells close together but not heaped up. It is allowed to stand in a warm room, and is inspected occasionally for an hour, if agglutination does not occur sooner. As a rule agglutination, if it occurs at all, will occur within 5 or 10 minutes and be unmistakable. If it is not definite, the mixture is agitated by gently tilting the slide from side to side, or a mechanical shaker is used. The cells will then nearly always settle out, conspicuously and compactly clumped. In preparations in which no agglutination occurs the cells will remain evenly distributed for hours.

With these data, the group can be determined quickly from the following outline. The occurrence of agglutination is indicated by +.

Agglutination in:

Group A serum	*Group B serum*	*Group O serum*	*Group of Cells Tested Is*
+	+	+	AB
0	+	+	A
+	0	+	B
0	0	0	O

On the basis of these tests, a donor belonging to the same group as the patient is selected, or if this is not possible, a Group O donor, and the following is set up:

4. Recipient's serum + donor's cells.

If this preparation shows no agglutination or hemolysis, the blood is compatible, and the donor may be used. In the case of patients who are receiving repeated transfusions and of all women who have not passed the child-bearing period, tests for the Rh factor should be made, and Rh-negative individuals should be given only Rh-negative blood.

Vincent's *open slide method*, recommended by Ottenberg, is preferred by many workers.

A full drop of serum and of cell suspension are mixed on a slide, preferably in the concavity of a hollow ground slide. Drying must be prevented, and this can be accomplished by covering the hollow with a coverslip and sealing with petrolatum. The slide is tilted and rotated gently so that the cells are uniformly distributed, and this is repeated every two minutes for at least 30 minutes, and preferably for an hour. A mechanical shaker may be used. The preparations are kept at room temperature, and are examined with the naked eye, not with the microscope. True agglutination is easily visible.

TUBE METHOD. The most sensitive and quickest method consists of placing in a small test tube one drop of serum, one drop of 1 to 2 per cent cell suspension and one drop of salt solution. One or more control tubes are prepared in a similar manner. The contents of the tubes are mixed and, after two to five minutes, centrifuged until the cells are lightly packed. The reaction occurs promptly and incubation is not necessary. The tubes are agitated gently until the cells in the control (negative) tubes are uniformly suspended. If any agglutination occurs, there are usually unmistakable macroscopic clumps visible. If there is doubt, a drop of the suspension may be examined microscopically.

In determining the blood group, the procedures should be carried out at room temperature (or in the incubator), not in the icebox, in order to avoid the effect of certain minor ("cold") agglutinins. The major agglutinins are also more active at low temperatures, but chilling is rarely necessary to demonstrate them. Anti-Rh agglutinins, however, are usually most active at 37° C. If their presence is suspected, to determine the major group, tests should be made at room temperature.

SOURCES OF ERROR. The chief sources of technical error are: (1) Weak or deteriorated sera. Their activity must be checked frequently by testing with known agglutinable cells. (2) Hemolysis, which may mask agglutination. The remedy is to inactivate the serum, or use stored serum. (3) Use of too thin or too thick a cell suspension. If an excessive quantity of cells is present, weak reactions may be missed. Hence the use of undiluted blood as a source of cells is not recommended. (4) Failure to agitate the suspension. (5) Confusion of rouleaux with agglutination. The microscopic appearance of the aggregates is usually quite different. Agitation breaks up rouleaux, but intensifies agglutination. Rouleaux formation rarely occurs in stored serum, and it can be prevented by slight dilution of the serum. Except as a source of confusion, rouleaux formation has no significance for transfusion. (6) Dependence upon the agglutinating activity of the serum being tested, which may be weak, instead of using stock sera of high titer. (7) Incubation at too low temperatures (see below). (8) The use of serum or cell suspensions which are badly contaminated by bacteria. (Nonspecific agglutination may occur.) (9) Rarely, the presence of anti-Rh agglutinins. (10) Gross errors in labeling and technic.

Errors resulting from failure to demonstrate agglutination when it should be present are most likely to occur with subgroups A_2 and A_2B (see p. 407). Cells containing A_2 are agglutinated with difficulty, and sera of high titer are required to identify them. Cells of infants of all groups are less agglutinable than those of adults and especial care is required in examining them. Their sera often lack agglutinin entirely, and this check on the accuracy of the routine tests is therefore not available.

If stock sera of Groups A and B are not available, the group of a patient may be determined by testing his cells and serum with those from either a known Group A, or a Group B individual, thus:

Serum A and X Cells	*Serum X and A Cells*	*Group Is*	*Serum B and X Cells*	*Serum X and B Cells*
+	0	AB	+	0
0	0	A	+	+
+	+	B	0	0
0	+	O	0	+

The disadvantage is that errors may occur if the agglutinating activity of either serum happens to be feeble.

If neither stock sera nor individuals of known group are available, in emergencies one must depend on preliminary "cross-matching" tests for the selection of a donor.

Dangerous Universal Donors. When a transfusion of 500 ml. is given to an adult of average size, the blood of the donor is diluted about 1 : 10 in that of the recipient. Very frequently the blood of a Group O donor shows an agglutinin titer substantially higher than 1 : 10. On the assumptions that reactions to incompatible blood are due to agglutination of red cells in the body, and that the usual absence of such reactions when Group O individuals are used as universal donors is due purely to dilution, many writers have emphasized the theoretical danger of using a universal donor whose serum has a high agglutinin titer, and have called them "dangerous universal donors." There is, as a matter of fact, no positive evidence that either of these assumptions is true. Furthermore, clinical experience indicates that reactions which can be ascribed to this rarely if ever occur in spite of the frequency with which universal donors have been used without regard to the titer of their serum. In none of the reported cases in which a transfusion reaction has been attributed to this incompatibility has the possible action of anti-Rh agglutinins been excluded. With massive transfusions, however, the risk is probably a real one, and even with transfusions of 500 ml. no reasonable precaution should be neglected. Experience indicates, however, that such blood may be safely used if the titer determined by the centrifuge-tube method is less than 1 : 500, provided the transfusion be administered slowly. Few sera will fail to show agglutinin in 1 : 10 dilution when tested in this way. The average titers have been given as 1 : 128 for A cells and 1 : 32 for B cells.

To test the titer, make a series of progressive dilutions of the donor's serum up to 1 : 1000. In a series of an equal number of small tubes put successively a drop of each serum dilution, and add to each one drop of 1 to 2 per cent cell suspension of the recipient. The contents of the tubes are mixed, centrifuged, and examined as described above. If any agglutination occurs in 1 : 500 or higher, another donor must be secured.

The agglutinating activity can be abolished *in vitro* and presumably *in vivo* by the addition of suitable amounts of purified A or B substance of Witebsky and Klendshoj (1941).

Preparation of Stock Serum. Sterile serum is secured from individuals known to belong to Group A, B, and (preferably also) O, whose serum has been shown to have active agglutinating power. One drop of sterile serum (enough for a single test) is sealed in each of a number of sterile capillary glass tubes. Overheating the serum while sealing must be avoided.

Or, to each 9 ml. of clear serum is added 1 ml. of 15 per cent sodium citrate solution in physiological salt solution. To each 9 ml. of this citrated serum, 1 ml. of 5 per cent solution of phenol in glycerin is added. Instead of phenol, 0.01 per cent acriflavin may be added to Group A serum and 0.01 per cent malachite green to Group B serum, both as a preservative and as a means of identification. Each serum should be labeled with care and handled separately to avoid accidental confusion of the groups. Such serum usually keeps well for several months in the icebox, but weakens more rapidly than if kept sterile without preservatives. If desiccated by the lyophile process, its activity is preserved for long periods.

Agglutinogens A_1 and A_2. Subgroups, in the sense of clear-cut subdivisions of the major blood groups, have been demonstrated only in the case of Groups A and AB. If properly selected Group B or Group O sera are titrated as to their agglutinative activity with cells from many Group A individuals, the latter are found to fall quite sharply into two subgroups. One possesses cells which are agglutinable in relatively high dilutions of serum, whereas the cells of the other are agglutinated only in relatively high concentrations. The titer for cells of the first subgroup may be four to eight times that for those of the second subgroup. Landsteiner et al. on the basis of absorption tests and other pro-

cedures attribute this and other differences between the subgroups to the possession of different agglutinogens in the cells, designating that in the readily agglutinable subgroup as A_1, the other as A_2. Subgroup A_1 is about four to five times as numerous as A_2. Serum from Group O and Group B usually contains two agglutinins active on A cells; one, known as anti-A, or α, agglutinates both A_1 and A_2 cells, about equally; the other, anti-A_1 or α_1, agglutinates only A_1 cells.

The simplest way to identify A_1 is to select a Group B serum with a high titer for A cells and absorb it repeatedly with known A_2 cells. This removes α agglutinin but leaves α_1 relatively intact. Cells which are agglutinated by such absorbed serum contain A_1, whereas cells from Group A which are not agglutinated are usually A_2. (In about five per cent of the cases the reactions are indecisive, for reasons which are not yet entirely clear.) By this means Group AB may be subdivided into A_1B and A_2B.

Agglutinogen A_2 is inherited as a Mendelian character, and it is believed to be transmitted by means of a fourth independent allelic gene which is dominant with respect to O, but recessive to A_1.

A third subgroup, A_3, has recently been identified, the cells of which are even more resistant to agglutination than A_2. It occurs in only about 1 in 1000 individuals.

In rare instances, A_2 (or A_2B) serum may agglutinate A_1 cells, and even more rarely A_1 or A_1B serum may agglutinate A_2 cells (α_2 agglutinin). The latter also agglutinates O cells, even more strongly than A_2 cells, and is sometimes called the anti-O agglutinin. These agglutination reactions are usually feeble, and demonstrable only by the most sensitive procedures at icebox temperatures.

In nearly all cases, individuals in the two different subgroups are compatible for purposes of transfusion, although in very rare instances transfusion reactions attributable to this discrepancy have occurred. A point of greater practical importance is that Group B sera differ much in their relative content of α and α_1 agglutinin. With a stock B serum weak in α agglutinin, an A_2 individual may be mistaken for one belonging to Group O and used as donor for a Group O patient with disastrous results. Group A_2B cells are poorly agglutinated by many B sera, and special care is required to avoid mistaking them for Group B cells.

Rh Factor. In rare instances patients who have received repeated transfusions of blood from donors of the homologous major blood group eventually exhibit severe hemolytic reactions although previous transfusions even from the same donor had caused no reaction. It has been shown that these reactions in most cases are due to the development in the recipient of antibodies for an agglutinogen in the cells of the donor which is different from A_1, A_2, B, M, and N. To this agglutinogen Landsteiner and Wiener applied the term "Rh" because the corresponding anti-Rh agglutinin was identical with one which they had produced in rabbits by injections of blood from rhesus monkeys. Using agglutinating sera from such patients or from immunized animals, the Rh agglutinable factor was demonstrated in about 85 per cent of the (white) individuals tested. In the 15 per cent who were Rh-negative, normally no anti-Rh agglutinin is demonstrable. It may appear (only) after immunization, either as a result of repeated transfusions of Rh-positive blood, or in pregnant women having an Rh-positive fetus. This occurs, however, in only about 2 to 4 per cent of the cases in which it might be expected.

In a woman so sensitized a first transfusion of Rh-positive blood may cause a hemolytic transfusion reaction. Antibodies from the mother may also gain access to the fetal circulation and cause erythroblastosis (see p. 452). For such sensitized patients it is imperative to have Rh-negative donors.

Blood sera taken from different sensitized individuals may differ qualitatively in their agglutinative action on various Rh-positive cells. Three specific types of agglutinating sera have been recognized: (1) the "standard" anti-Rh serum (anti-Rho) which agglutinates the red cells of 85 per cent of the individuals tested; (2) a type (anti-Rh′) which agglutinates the cells of 70 per cent; and (3) a third (anti-Rh″) which agglutinates the cells

of only 30 per cent. (There are between 1 and 2 per cent of individuals who are Rh-positive, but in whom this is not detected by use of the first type of serum.) Certain sera may contain two agglutinins, e.g., anti-Rho and anti-Rh′, or anti-Rho and anti-Rh″.

A detailed study of these reactions, particularly by Wiener, Levine, Race, and their associates, indicates that there are three different "Rh factors" corresponding to the three agglutinins above mentioned. These factors occur singly or in combination to form at least five different agglutinogens which have been designated as follows: Rh_0, Rh′, Rh″, Rh_1 (= Rh′$_0$), and Rh_2 (= Rh″$_0$). By means of the three types of agglutinating sera, eight different "Rh groups" or types have been distinguished. The designations and frequency of these types in the white population have been given by Wiener as follows: Rh_1, 54.5 per cent; Rh_2, 15 per cent; Rh_1Rh_2, 13 per cent; Rh_0, 2.5 per cent; Rh′, 1.2 per cent; Rh″, 0.3 per cent; Rh′Rh″, 0.01 per cent; Rh-negative, 13.5 per cent. The frequency of the types is different in other races. Rh-negative cases are quite rare in Mongolians. These conclusions in the main are in harmony with those of the British investigators, but the latter use an entirely different nomenclature.

The Rh agglutinogens are inherited as dominant Mendelian characters, independently of the other isoagglutinins. This is accomplished, according to Wiener, by means of six allelic genes, of which five correspond to the five agglutinogens above listed, and the sixth, rh, is a recessive character which determines Rh-negative individuals. Each somatic cell is supposed to possess two genes, one derived from each parent, and each maturated sex cell, one gene. Fisher has advanced a different theory, based on the work of the British group, for which there is strong supporting evidence. The Rh type is determined by three factors, each of which exists in at least two alternative forms, C or c, D or d, and E or e. Each cell carries three genes, one determining each of these factors. Thus Wiener's Type Rh_1 corresponds to Cde, Rh_1Rh_2 to CDE, and rh to cde. New types of blood, the existence of which was predicted on the basis of this theory, have since been discovered.

Hr Factor. In about 8 per cent of the cases the mothers of infants with erythroblastosis have been found to be Rh-positive. Levine showed that (in about one-third of these cases) this may be explained by assuming the presence in the fetus of a factor analogous to Rh but different from it, to which he gave the name "Hr." The corresponding agglutinin, present in the serum of the mother, he termed "anti-Hr" because in its activities it was the reciprocal of the anti-Rh′ agglutinin. Anti-Hr sera therefore agglutinate (among others) the cells of Rh-negative individuals. According to Race and Taylor, the Hr factor is determined by the presence in the cells (of the fetus) of certain of the Rh factors (rh, Rh_2, Rh″ and Rh_0). If these factors are absent in the mother (who must possess other Rh factors, since she is Rh-positive), she may become sensitized to them, just as if she were completely Rh-negative. The infant is Rh-positive. In addition to this anti-Hr′ serum, Race and Taylor postulated the existence of anti-Hr″ and anti-Hr$_0$ sera, and the former has since been identified.

These relationships are manifestly intricate, and some of the points discussed are still controversial. Recent work also indicates that there may be additional Rh factors which would determine the existence of other Rh types. These, however, would necessarily be rare.

Testing for Rh Factor. To test for the Rh factor, for practical purposes it suffices to use a standard anti-Rho serum which gives positive reactions with 85 per cent of random bloods, using as potent a specimen as is obtainable. Such serum may be obtained from sensitized patients, preferably mothers of erythroblastotic infants. Practical difficulties arise in obtaining suitable serum from the relative rarity of such cases. Furthermore, many sera give weak agglutination reactions *in vitro*, and they tend to weaken rapidly *in vitro* and *in vivo*, and may become useless within a few weeks. Isoagglutinins if present must be removed or neutralized with specific A and B substance. (Suitable serum for routine use may now be purchased from Certified Donor Service, 146-16 Hillside Ave., Jamaica, New York.)

PROCEDURE. (1) The serum is inactivated at 56° C. for 15 minutes. (2) The cells from each patient to be tested are washed, and a 2 per cent suspension is made in 0.85 per cent salt solution. (3) Set up in a rack a series of four small 7-mm. tubes for each blood to be tested, including controls of known Rh-positive and Rh-negative blood. (4) In the first two tubes of each set place 0.2 ml. of standard Rh agglutinating serum. (5) In tubes 2, 3, and 4, place 0.2 ml. of salt solution. Dilution is accomplished by mixing the contents of tube 2, transferring 0.2 ml. to tube 3, and continuing thus, discarding 0.2 ml. from tube 4. (If commercial serum is used, dilution is unnecessary and a single tube suffices.) (6) To each tube add 0.1 ml. of the appropriate cell suspension; after mixing the contents the tubes are placed in a water bath at 37° C. for an hour. (7) Without shaking, the tubes are inspected with the aid of a hand lens. In negative reactions the sediment has an even surface and a smooth border. A positive reaction is indicated by a more or less granular or wrinkled surface and an irregular margin. After shaking the tubes gently, one drop is removed from each and examined microscopically for clumps. It is often impossible to distinguish clumps macroscopically. (8) The tubes are allowed to stand for two hours and again inspected. These reactions are usually much feebler than those caused by the usual isoagglutinins, and care and experience are needed to interpret them correctly.

To test a serum for anti-Rh agglutinin, the same procedure is followed, except that known Rh-positive cells are used. If, however, the reaction at the conclusion of step 8 is negative, in order to detect blocking antibodies if present (see below), (9) pipet off the supernatant fluid, add an equal volume of homologous serum or albumin solution, and mix. If antibody is present, clumping will occur within a few minutes at room temperature.

With a large majority of sera, these reactions are most active at body temperature. In rare instances they are more active at room or even icebox temperature, and if a negative reaction is unexpectedly obtained, the tubes should be shaken, allowed to stand at a lower temperature and again inspected.

BLOCKING ANTIBODIES. In a substantial number of patients in whose blood anti-Rh agglutinins would be anticipated, agglutinating activity cannot be demonstrated by this procedure. If, however, Rh-positive cells in suitable quantity are added to such a serum and subsequently removed by centrifugation and washed free from serum, they will no longer be agglutinable if added to an active anti-Rh agglutinating serum. They appear to have absorbed from the original serum a specific "incomplete" antibody, which has been termed a blocking or coating antibody because it prevents subsequent combination with an active or "complete" agglutinin.

If, however, Rh-positive cells are suspended in homologous serum or in a 20 per cent solution of human or bovine albumin and serum containing antibody in either form is added, the cells will be quickly and coarsely clumped. This phenomenon, known as conglutination, is brought about by an additional substance ("X protein," "glutinin") present in serum but inactivated by slight dilution. The following simple test is based on these observations.

SLIDE TEST (Diamond and Abelson, 1945). (1) Make a 1 to 2 per cent suspension of Rh-positive cells in albumin solution or in homologous (or compatible) serum. (2) Place 0.2 ml. cell suspension on a slide and add an equal volume of the serum to be tested. (3) Warm gently over an electric light bulb, tilting the slide gently, and observe for three minutes. If antibody is present, macroscopic clumping will occur. A similar preparation containing Rh-negative cells should be set up as a control.

Minor (Cold) Agglutinins. In certain cases the serum of one individual may agglutinate the cells of another belonging to the same blood group, or to Group O, even if the action of anti-Rh agglutinins is excluded, provided the mixtures are kept at icebox temperature. These reactions are dependent upon the interaction of several different agglutinin-receptor pairs, only one of which, P-p, has been adequately studied. Their

incidence appears to be entirely independent of that of the four major groups. As a rule this agglutination is feeble, it occurs only in high concentrations of serum, and it disappears if the preparations are warmed. Occasionally, however, the agglutination is more marked, and it may then cause errors in group determination. Transfusion reactions due to this type of incompatibility appear to be very rare if they occur at all.

Autoagglutination (Cold Agglutination). If mixtures of serum and cells from the same individual are kept at icebox temperature, in some cases agglutination occurs. The clumps break up as soon as the mixtures are warmed. Such serum will similarly clump any red cells (human or animal) added to it; i.e., it is a *panagglutination*. Slight degrees of such agglutination are said to be common, but a marked degree of agglutination is infrequent. It occurs, however, in a considerable number (not all) of the cases of primary atypical pneumonia, and less frequently in other acute infections, particularly of the respiratory tract. It has been reported in acquired hemolytic jaundice, in cirrhosis and other diseases of the liver, in chronic sepsis, leukemia, pernicious anemia, etc. The reaction is therefore not specific. It is of some value empirically in the diagnosis of primary atypical pneumonia, but otherwise has little if any practical significance.

Acute hemolytic anemia has been observed in patients whose blood contained cold agglutinins in high titer. Some have attributed this to intravascular agglutination resulting from chilling of the extremities.

Procedure. To determine the titer of the agglutinin, the blood must be kept warm, preferably at 37° C., until clear serum has separated and been pipetted from the clot. (1) Into each of a series of 10 small test tubes pipet just 0.2 ml. physiological salt solution. (2) To the first tube, add 0.2 ml. clear serum. After mixing, transfer 0.2 ml. of the contents of this tube to the second tube. In a similar way, progressive dilutions are made in each tube, finally discarding 0.2 ml. from the last tube. (3) Prepare a 1 per cent suspension of washed red cells, either from the patient, from another individual belonging to the same major blood group, or from an individual of Group O. (4) To each tube add 0.2 ml. of red cell suspension. After mixing the contents the tubes are placed in the icebox for two hours or overnight. (5) They are then removed and read at once. After warming to 37° C. they are examined again to make certain that the clumping has broken up.

The exact titer recorded with a given serum will vary, depending upon the exact technic used and the observer's criteria of a positive reaction. Observations may be made macroscopically or microscopically. In microscopic examination the tubes are shaken thoroughly and a drop of the contents of each is inspected under the low-power objective. Read in this way, the titer is two to four times higher than if done macroscopically. The titer after standing 16 to 20 hours is two to four times higher than if read after two to three hours. Spingarn and Jones (1945), using a slightly different technic and making readings microscopically after 16 to 20 hours, concluded that titers less than 1 : 112 were of no significance. Of 100 normal controls, 89 showed titers less than this and none over 1 : 224. Of 91 patients with primary atypical pneumonia, 56 showed titers of 1 : 112 or higher, and 40 over 1 : 224. Substantially equal titers were observed, however, in occasional cases of other acute infections, including measles, mumps orchitis, infectious mononucleosis, and scarlet fever.

The autohemolysin of paroxysmal hemoglobinuria is described in a following chapter.

Medicolegal Applications

Disputed Parentage. Since the receptors A and B are inherited as independent dominant Mendelian characters, it follows that *a receptor cannot appear in a child unless it is present in one of the parents.* Therefore, if the group of the child and of one parent is known, it is possible in some cases to determine that a given individual cannot be the other parent. This subject has been further elaborated by Bernstein (1924), who advanced the theory that the inheritance of the blood groups is determined by the distribution of three allelomorphic genes: A, B, (dominants), and R (or O), a recessive (negative). One

gene is present in each member of a pair of chromosomes, so that each somatic cell possesses two of these genes, and each sex cell, one gene. The genetic structure of the four groups would, therefore, be: Group O, OO; Group A, AA or AO; Group B, BB or BO; Group AB, AB. Since a child draws one gene from each parent, it follows that a Group O parent can never have a Group AB child, and a Group AB parent can never have a Group O child, regardless of the group of the other parent. With any other combination of groups in the parents, a child may have O genes only, or either A or B or both, provided these are present in either parent. Recent careful studies of large numbers of families have confirmed these assumptions, and their validity has been upheld in the courts in many European countries. In this country they have been used to a limited extent. Definite information is obtained in from 20 to 30 per cent of the disputed situations.

Landsteiner et al. (1928) have demonstrated two other definite agglutinogens in human blood, which they have designated as M and N. There are no corresponding agglutinins in human serum, and their demonstration is a highly complicated procedure requiring the blood of specially immunized animals. Their distribution is entirely independent of that of the four major groups. One or both of them are present in the blood of every human being. Like agglutinogens A and B, M and N are inherited as independent dominants, and human beings can be divided on this basis into three classes or "groups," having a theoretical genetic structure of MN (50 per cent), MM (30 per cent), and NN (20 per cent). The presence of these agglutinogens can also be utilized to decide questions of disputed parentage. This gives definite information in about an additional 30 per cent of the cases.

Determination of the Rh type has also been utilized for this purpose as well as the presence or absence of the factor, P. By employing all of these procedures it is possible theoretically to differentiate 288 varieties of human blood.

Determination of the Group in Blood Stains. If the blood dried before decomposition occurred, and if the proteins have not been denatured by heat or otherwise, it is usually possible to determine the group of the individual from whom the spot was derived. It is useless to attempt direct agglutination of cells recovered from such material, and only occasionally is it possible to demonstrate agglutinins directly in extracts of such spots. The only practicable method is based upon the fact that the isoagglutinins are specifically "absorbed" and removed from a serum when brought into contact with red cells containing the corresponding receptor, or with the products of hemolysis of such cells, or with seminal fluid or tissue juices of individuals belonging to the same group.

Method. (1) A serum and B serum with a high agglutinin titer (preferably 1 : 160) are secured, and each is diluted five times with salt solution.

(2) Into each of two centrifuge tubes are placed 20 to 40 mg. of dried blood, or a piece of stained cloth (if heavily blood-stained 1 sq. cm. suffices) is cut into fine bits, and a half placed in each tube. (3) To each tube is added 0.5 ml. salt solution; the tubes are allowed to stand for one hour. (4) To tube 1, 0.5 ml. diluted A serum is added, and to tube 2, 0.5 ml. diluted B serum; the tubes are then allowed to stand for three hours. (5) They are centrifuged, the clear fluid which contains serum in 1 : 10 dilution is removed, and a series of dilutions of each, in 0.5 ml. volume, from 1 : 10 to 1 : 160, is set up. (6) To each tube in the first series (containing A serum) 2 drops of a 5 per cent suspension of washed Group B cells are added; to each tube in the second series a similar quantity of A cells is added. (7) The reaction is read after two hours.

If agglutination occurs as indicated, the group of the blood in the spot is:

A Cells	*B Cells*	*Group Is*
+	0	B
0	+	A
+	+	O
0	0	AB

To ensure the certainty in the results needed for medicolegal purposes, elaborate controls are necessary, including parallel absorption tests with blood spots of known groups. By this method the group has been determined in blood spots 15 to 20 years old.

Substitutes for Blood

To replace blood lost by acute hemorrhage, the best fluid is whole blood, and the most satisfactory substitute is human plasma. In surgical shock without hemorrhage, plasma is equally as effective as whole blood. Plasma is often life-saving in such conditions, since the cause of death is not lack of erythrocytes but insufficient fluid to fill the blood vessels adequately and maintain blood pressure. Plasma may be preserved and administered in fluid form, or, for the sake of convenience in storage or transportation, it may be desiccated by a lyophile process and redissolved in sterile distilled water just before administration. No satisfactory substitute for human plasma has yet been found.

Physiological salt solution, Locke's solution, glucose solution, or a solution of amino acid mixtures may be administered safely, and these are useful in combating dehydration and maintaining nutrition, but they are ineffective in shock because both the solute and the fluid are eliminated with great rapidity.

Many attempts have been made to find a colloidal solution which will be retained within the vessels and which might be used if neither blood nor plasma were available. A solution of gum acacia in physiological salt solution can be given without serious immediate reaction if properly prepared, and is relatively effective, since the acacia is eliminated slowly and the fluid is retained in the vessels. This has been largely abandoned, however, since it was found that the acacia is taken up by the tissue cells and stored for long periods. Among other materials which have been tried may be mentioned gelatin, pectin solutions, and albumin from horses or cattle which has been highly purified and (in some cases) "denatured" in various ways. If given repeatedly, however, the latter tends to cause sensitization and serious foreign-protein reactions.

Preservation of Blood for Transfusion

Blood which has been drawn into sodium citrate solution (70 ml. of a 2.5 per cent solution per 500 ml. of blood) may be preserved for some time by storing it in the icebox at 4° C. The addition of 20 to 30 ml. of 10 per cent glucose solution markedly delays deterioration of the red corpuscles. The group should be determined, a serologic test for syphilis carried out, and cultures made to prove sterility. Before use, a cross-matching with the blood of the prospective recipient should be carried out. Although blood has often been used after storage for four weeks and more, it gradually deteriorates with age, and it is probable that 10 to 14 days is about the maximum limit during which it can be used with perfect safety and optimum results. According to Bushley et al. (1940), red cells in blood (containing added glucose) which has been stored 14 days survive *in vivo* practically as long as those in fresh blood. As blood platelets practically disappear after about four days and prothrombin also diminishes, fresh blood should be used to supply a deficiency of these constituents. The plasma from overaged blood may be siphoned off and used safely if no hemolysis has occurred.

Strumia et al. (1947) recommend the following preservative (B. M. H. A. C. D. No. 3). For each 500 ml. blood use 75 ml. distilled water containing 1.6 Gm. sodium citrate, 0.56 Gm. monohydrated citric acid, and 1.5 Gm. anhydrous glucose. This is said to preserve the cells in excellent condition for three weeks.

If red cells only are needed and not plasma, it may be desirable to remove the plasma and resuspend the cells in some neutral medium. Thalheimer and Taylor (1945) recommended for this purpose a 10 per cent solution of corn syrup in distilled water. They reported that in this medium red cells remain in excellent condition for two to three weeks. Strumia et al. (1947) report that the red cells are better preserved in a medium

consisting of distilled water containing 2.4 per cent anhydrous glucose, 1.9 per cent sodium citrate and 4 per cent globin.

Cadaver Blood

Yudin and his associates at Moscow (1932, 1937) have reported the successful use of blood obtained from cadavers within six to eight hours after death. Blood (2 to 3 liters) was drawn aseptically from the jugular vein. It might be mixed with citrate solution in the usual way. If, however, the blood was obtained from individuals who had died suddenly, either from accidents or from such conditions as coronary thrombosis, and was allowed to coagulate, the clot would speedily undergo fibrinolysis, and the re-liquefied blood would not again clot. A serologic test for syphilis, cultures, and a complete necropsy were carried out to exclude conditions which might contraindicate its use. (In warm climates, thick blood films and films from the spleen and bone marrow would also be required, to exclude malaria.) They found that such blood could be preserved for three to four weeks by storing it at 1° to 2° C. It was as effective and caused fewer febrile reactions (5 per cent as compared with 20 per cent) than fresh citrated blood from living donors.

CHAPTER 14

Normal and Pathologic Blood Cells

Red Cells

Normal Values. The normal values for red cells of young adults are given in the following table (slightly altered from Wintrobe).

Table 45

NORMAL VALUES FOR RED CELLS OF YOUNG ADULTS

	Male		*Female*	
	Average	*Range*	*Average*	*Range*
Red cell count in millions	5.4	4.6 to 6.2	4.8	4.2 to 5.4
Hemoglobin in Gm. per 100 ml.	15.6	14 to 18	14.2	12 to 16
Volume of packed cells per 100 ml.	47	40 to 54	42	37 to 47

	Average	*Range*
Mean corpuscular volume in cubic microns	85	82 to 92
Mean corpuscular hemoglobin in micro-micrograms	29	27 to 31
Mean corpuscular hemoglobin concentration in %	34	32 to 36

The red cell count in young infants is usually about 5.5 million. It falls rapidly during the first few weeks to about 5.0 million, and reaches a minimum (about 4.5 million) at one year. During childhood it remains somewhat lower than the adult figures. The red cell count may fluctuate during the day within a range of at least 10 per cent, with changes in plasma volume, or with the storage of red cells in, or their release from the spleen and other reservoirs.

The blood of the normal young male adult in this country has an average O-combining capacity of 20.9 volumes per cent (Van Slyke), corresponding to 15.6 Gm. hemoglobin per 100 ml. In Great Britain the normal figure is usually given as 14.5 Gm. The variations with age and the daily fluctuations parallel those noted for the red cell count.

For convenience in calculating the indices it is customary to regard 5.0 million red cells per cu. mm. as the normal figure, and the quantity of hemoglobin in normal blood having exactly this red cell count as the normal figure for hemoglobin (Osgood's hemoglobin coefficient). On this basis 14.5 Gm. hemoglobin corresponds to 100 per cent. Similarly, the average volume of packed red cells in normal blood having a red cell count of 5.0 million per cu. mm. is 42 per cent (the volume coefficient of Osgood).

The *mean corpuscular volume* (Wintrobe) in cubic microns is calculated by dividing the volume of packed red cells, expressed in milliliters per liter, by the red cell count in millions. The *mean corpuscular hemoglobin* in micro-micrograms ($\gamma\gamma$) is calculated by dividing the hemoglobin content of the blood, expressed in grams per liter, by the red cell count in millions. A micro-microgram is one millionth of one millionth of a gram. The *mean corpuscular hemoglobin concentration* in per cent is calculated by dividing the hemoglobin content of the blood, expressed in grams per liter, by the volume of packed

red cells, in milliliters per liter, and multiplying by 100. These are absolute values, and not dependent numerically on any arbitrary standard of normal.

These relationships are more commonly expressed as *indices*.

The *color index* (color-count ratio) expresses the relative amount of hemoglobin in each red cell, as compared with that of normal blood. It is obtained by dividing the hemoglobin content of the blood by the red cell count, both expressed in percentages of the normal figures. The percentage of the normal red cell count is obtained simply by multiplying the first two figures of the count by two.

The *volume index* (volume-count ratio) expresses the relative volume of the individual red cell, as compared with that of normal blood. It is calculated by dividing the red cell volume by the red cell count, both expressed in percentages of the normal figures.

The *saturation index* (color-volume ratio) expresses the relative concentration of hemoglobin in the red cells, as compared with normal blood. It is calculated by dividing the hemoglobin by the red cell volume, both expressed in percentages of normal. It may be diminished, but it is never increased.

The average normal value for each index is, by definition, 1.0, and the normal range is at least from 0.9 to 1.1. The great importance of these relationships in the classification and diagnosis of anemias is discussed in the following chapter.

EXAMPLE. The normal values commonly used for these calculations are: Red cell count, 5.0 million. Hemoglobin, 14.5 Gm. per 100 ml. Cell volume, 42 per cent. Assume that a given blood shows:

Red cell count 2.5 million = 50 per cent of normal.
Hb. is 8.7 Gm., = 60 per cent of normal.
Cell volume is 30 per cent, = 71 per cent of normal.
Then the color index = Hb./count = $^{60}/_{50}$ = 1.2
The volume index = Volume/count = $^{71}/_{50}$ = 1.4
The saturation index = Hb./volume = $^{60}/_{71}$ = 0.86
The mean corpuscular volume = 300/2.5 = 120 cubic microns
The mean corpuscular Hb. = 87/2.5 = 35$\gamma\gamma$
The mean corpuscular Hb. concentration = 100 × $^{87}/_{300}$ = 29 per cent

Morphology of the Red Cells. In normal blood the red cells are biconcave discs, all circular in outline and nearly uniform in size, with a mean diameter of about 7.5μ (7.2, Price-Jones) and a mean thickness of 1.9 to 2μ. Two thirds of the cells are from 7 to 8μ in diameter. An occasional cell may be as small as 5.5μ, or as large as 9.5μ. A greater diversity in size is called *anisocytosis*. Cells which are less than 5μ in diameter are termed *microcytes;* those over 10μ, *macrocytes*. Cells which are abnormal in shape, like a sausage, battledore, or dumbbell, *poikilocytes*. Most of the microcytes and poikilocytes are probably portions of fragmented cells.

With or without marked diversity in size, the average size of the red cells may be abnormally small (*microcytosis*) or abnormally large (*macrocytosis*). The determination of the mean corpuscular volume or volume index gives a better indication of the average size of the cells than merely a measurement of their diameter, but in most conditions there is a rough parallelism between them.

The depth of color of the cells, either in fresh or stained films, is nearly as constant as their size. Abnormal variations in color (*anisochromia*) are usually due to variations in thickness of the cells. Unusual pallor of the cells (*"achromia"*) may be due in part to

diminished concentration of hemoglobin in the cell. Such cells may have colorless centers with only a narrow yellowish rim of hemoglobin-containing stroma ("pessary forms").

In cells which show true achromia, there is a gradual transition in depth of color from a pale center to a more deeply stained periphery. This must be distinguished from a common type of artefact which is produced during drying and fixation of the film and which is characterized by a colorless round central area sharply demarcated from a dark peripheral rim. The latter is prone to occur in the thin cells in patients with hypochromic anemia, but it may also occur in normal blood and has only restricted (if any) diagnostic significance.

Immature Red Blood Cells. These are found in any form of anemia in which there is active regeneration of new cells. They may be regarded as hurried into the circulation before development is completed, to satisfy an urgent need for cells.

Polychromatophilia, Polychromasia, Diffuse Basophilia. In films stained with any type of Romanowsky stain a limited number of the red cells may show a more or less marked degree of diffuse bluish staining. The term *punctate basophilia* or *stippling* (Grawitz granules) is applied to red cells showing discrete, bluish-black granules with such stains. They are especially numerous in lead poisoning. Unlike reticulum they can be seen in fresh preparations. The granules are regarded as evidence of degenerative changes in the cytoplasm of immature cells.

Reticulocytes. Reticulocytes in increased number constitute the most constant and dependable sign of active regeneration in anemia of any type. They are a little larger than mature erythrocytes. In vitally stained films they contain bluish granules which, in the more immature cells, are numerous and connected with one another by a meshwork of fine bluish strands. The more mature reticulocytes contain a few similar granules, or even only a single granule, without any visible strands, and are visible only in the best preparations. The granules are generally considered to be remains of the spongioplasm of the erythroblast. It is believed that they disappear within a few days as the cells mature in the circulation. They are not identical with the granules in ordinary stippled cells. Reticulocytes are more numerous than stippled cells, and may be numerous in blood which contains no stippled cells. An abrupt rise to a peak of 5 to 40 per cent or more, a *reticulocyte crisis,* occurs within a few days after the institution of effective treatment in severe cases of pernicious anemia or hypochromic anemia. Reticulocytes are especially numerous in familial hemolytic jaundice (frequently 20 per cent, rarely even 50 per cent).

Nucleated Red Cells (Erythroblasts). *Normoblasts* are approximately the size of a normal red cell or slightly larger. The more nearly mature forms contain a nucleus which has a diameter from one-half to two-thirds that of the cell, which is round, and which stains a dense, homogeneous, purplish-black color, without showing any structure (pyknotic). The nucleus may be lobulated or fragmented. The cytoplasm may be normal (orthochromatic normoblasts) or basophilic in varying degree (polychromatophilic normoblasts). The *"immature normoblasts"* (erythroblasts of some hematologists) tend to be somewhat larger and have a more basophilic cytoplasm. The nucleus shows definite structure and usually contains coarse, deeply stained, chromatin granules which are often clumped in wedge-shaped masses separated by radiating, pale lines, which give it a "wheel-spoke" appearance.

The more primitive erythroblasts (*"macroblasts"*), seen only in severe anemias, have nuclei with a finer chromatin structure, but except in the most primitive cells the strands are relatively thick; there is a tendency to clump, and the wheel-spoke arrangement of the chromatin often shows more or less definitely. Nucleoli are present. The cytoplasm is abundant and is strongly basophilic, so that it may be difficult to distinguish these cells from primitive leukocytes.

Megaloblasts are the most primitive red cells which appear in the circulation. They are large cells, 10 to 20μ or more in diameter. The nucleus is about as large as a normal red cell, it is approximately central, and there is usually no pale perinuclear zone. It shows

a nuclear membrane and contains interlacing strands of fine chromatin granules. The most primitive cells ("promegaloblasts") are even larger (up to 30μ), with a network of fine, evenly distributed chromatin granules and 3 to 5 nucleoli. Rarely mitotic figures may be found. The cytoplasm stains intensely blue without any suggestion of hemoglobin, but in slightly more mature cells it often has a greenish tinge. It looks waxy, homogeneous, and uniformly thick out to the extreme margin of the cell. This point helps to distinguish megaloblasts from primitive leukocytes which usually have thinner looking, somewhat irregular margins. In somewhat more mature cells the cytoplasm becomes polychromatophilic and the chromatin coarser, but there is little tendency to the clumping seen in the normoblastic series, and no wheel-spoke pattern. In the more mature megaloblasts the cytoplasm becomes orthochromatic, and the nucleus may become pyknotic, so that except for their larger size they are scarcely distinguishable from normoblasts. These cells rarely if ever occur in the blood except in severe untreated cases of pernicious anemia. The view of Ehrlich, Naegeli, and others, that megaloblasts in this restricted sense are pathognomonic of pernicious anemia (and related anemias) and are fundamentally different from the erythroblasts seen in other diseases has, we believe, much to recommend it, but it is not universally accepted.

Primitive erythroblasts are nonmotile. They contain many coarse, rod-shaped or coccoid mitochondria, but no inclusions which take up neutral red in supravitally stained preparations.

Nuclear particles differ from the granules previously described in that they take a purplish-red color with Wright's stain. They may occur as fine granules, usually clustered about the periphery of the cell, *chromatin dust*. When they are in the form of coarse granules they are called *Howell-Jolly bodies*. They may also occur as filaments which are variously looped or curled (*Cabot's rings*). These structures may be found in any condition in which nucleated red cells are present and have about the same significance. They are usually sparse, but may be numerous for many months after splenectomy, even in the absence of anemia.

White Cells

The *total leukocyte count* in normal blood ranges from 5000 to 10,000 per cu. mm. Variations as great as this may be observed in the blood of the same normal individual from day to day, or at different hours of the same day.

The leukocytes are made up of three distinct groups of cells which differ from one another fundamentally in origin, in structure, and in function. These are the granular (myeloid) leukocytes, or granulocytes, the lymphocytes, and the monocytes.

The appearance of the cells in fresh, supravitally stained films has been described in the previous chapter. The following descriptions apply to films stained by Wright's or Giemsa's method.

Granulocytes. The granulocytes contain definite granules which are visible in fresh as well as in stained preparations. In normal blood there are three varieties, distinguished by the staining reaction of the granules: neutrophils, eosinophils (acidophils), and basophils.

The *polymorphonuclear neutrophils* show a faintly pinkish-tinged cytoplasm filled with nearly uniform, fine granules which take a pink, lilac, or (with alkaline stains) a bluish-violet color. The nucleus is usually divided irregularly into from two to five lobes which are connected by thin filaments of chromatin. There is a distinct nuclear membrane. The chromatin is arranged in coarse strands which stain a deep purplish-blue color. There are

no nucleoli. In about 4 per cent of these cells the nucleus is band-shaped, or segmentation is incomplete, but the chromatin is in coarse strands and is densely stained. These are the "nonfilament" forms of Cooke and Ponder, and the Stabzellen of Schilling.

The *polymorphonuclear eosinophils* are distinguished by the coarse, uniform, highly refractile granules which stain a bright vermilion-red color. The nucleus is usually bilobed, larger, and finer in structure, and stains less intensely than that of the neutrophils.

The *polymorphonuclear basophils* (mast cells) contain purplish or bluish-black granules which are usually intermediate in size between those of the preceding types of cells, and are less refractile than the eosinophil granules. They tend to vary in size and depth of staining, and are often sparse. The nucleus stains more faintly, and the lobulation is often indistinct.

Immature granulocytes, the normal ancestors of the preceding types of cells, may appear in the blood in certain pathologic conditions. Normally some mechanism effectively prevents the passage of cells into the blood before their development is complete. It is the phenomenon of their entrance into the blood stream, and not the character of the cells themselves, which is pathologic. In general the more numerous and the less mature these cells are, the more serious is the disturbance of the hematopoietic tissue.

Myelocytes are the immediate precursors of the granulocytes. The more nearly mature forms resemble the mature granulocytes in their cytoplasm and specific granulations. There are neutrophilic, eosinophilic, and basophilic myelocytes. They differ from the mature cells in having round or oval nuclei, with little or no indentation. The nucleus takes a paler stain, and shows a finer chromatin network. There are no nucleoli. The myelocytes are nonmotile and not phagocytic in ordinary moist films, an important point in distinguishing them from monocytes. In suitable hanging-drop preparations, however, they show active motility, like that of the myeloblasts.

Metamyelocytes are cells which are intermediate in development between myelocytes and mature neutrophils. The nucleus has a moderately fine chromatin network, and is deeply indented, or has the shape of a broad band (the Jugendformen of Schilling). An occasional cell of this type may rarely be found in normal blood.

The *less mature myelocytes* differ in having a bluish rather than a pinkish cytoplasm, although a small area next to the nucleus may be pinkish. The granules are not differentiated, and take a bluish stain, or in the same cell some may be lilac or red, and some blue. They are relatively scanty. The nucleus has a fine chromatin network, and may contain nucleoli. The cells are often much larger than a mature granulocyte. The most primitive myelocytes, the *promyelocytes* (A-myelocytes of Sabin), have a deeply basophilic cytoplasm, and contain only a few (or even a single) bluish granules, or small purplish-red rods (Auer bodies) which take the peroxidase stain. The nucleus is like that of the myeloblast.

The *myeloblast* is the earliest recognizable precursor of the myelocytes. It is rarely seen in the blood except in the acute stages of leukemia. The cell is usually large, and has a large, somewhat eccentric, round or oval nucleus. This has a fine chromatin network which takes a reddish-purple color and gives the nucleus a velvety or finely stippled appearance. There are usually several (two to five) nucleoli, variable in size and shape, and pale sky-blue in color. They show no definite limiting membrane, and look like irregular lacunae in the chromatin network. There is no distinct nuclear membrane. Exceptionally the nucleus may be indented, convoluted, or irregularly lobulated. The cytoplasm is deeply basophilic, darker than the nucleus, and (by definition) contains no granules. There is no perinuclear clear area. In hanging-drop preparations, but not in ordinary moist films, they show a characteristic snail-like or wormlike movement.

Caption for Plate II

(*1*) Polymorphonuclear neutrophil.

(*2*) Polymorphonuclear basophil. The lobes of the nucleus are indistinct and are partly overlaid with granules. The latter vary in their size and depth of staining.

(*3*) Polymorphonuclear eosinophil. The granules are large and uniform in size.

(*4*) Clump of normal platelets.

(*5*) Small lymphocyte, with a wheel-spoke arrangement of the chromatin in the nucleus. There is a definite clear zone in the cytoplasm around the nucleus.

(*6*) Small lymphocyte, containing azure granules. The chromatin is in large masses.

(*7*) Large lymphocyte, containing azure granules. The chromatin is in coarse masses. There is an indistinct nucleolus. There is a pale zone in the cytoplasm around the nucleus.

(*8* and *9*) Monocytes. The cytoplasm is grayish blue in color. There is a fine lilac granulation which is abundant but does not stuff the cell as does that of the granulocytes. The nuclear chromatin is arranged in a fine network as contrasted with the coarse skeins of the granulocytes and the dense masses of the lymphocytes. There are no nucleoli. *9* is the transitional of Ehrlich.

(*10*) Plasma cell. The cytoplasm is abundant, basophilic, with two vacuoles and a small, pale, perinuclear zone. The chromatin masses are coarse and show the wheel-spoke arrangement.

(*11*) Giant platelet, from a case of pernicious anemia.

(*12*) Neutrophilic metamyelocyte, with band-shaped nucleus.

(*13*) Neutrophilic myelocyte, with oval nucleus and practically mature granulations.

(*14*) Primitive type of myelocyte, with sparse, bluish, undifferentiated granules. The cytoplasm is diffusely basophilic except for a narrow acidophilic zone at the left of the nucleus. The nuclear structure is fine, and there are two nucleoli.

(*15*) Myeloblast. There is no distinct nuclear membrane, the background of the nucleus is finely stippled, and there are five nucleoli. The cytoplasm is deeply basophilic, is free from granules, and shows no pale perinuclear zone.

(*16*) Premyelocyte. Essentially identical with the myeloblast except for a few reddish rod-shaped structures (Auer bodies) and bluish granules.

(*17*) Lymphoblast. The cytoplasm is deeply basophilic, with a perinuclear pale zone. There is a definite condensation of chromatin in the nuclear membrane and around the two nucleoli. The chromatin is appreciably coarser than in the myeloblast.

(*18*) Giant neutrophilic leukocyte, with hypersegmented nucleus from a case of pernicious anemia.

(*19*) "Toxic" neutrophil, from a patient with a severe infection, showing degenerative changes. The nucleus is swollen, with loss of the finer markings. The cytoplasm is vacuolated, and the granules are sparse, and variable and abnormal in size and tint.

(*20*) Rieder cell. A primitive lymphocyte with basophilic cytoplasm and a convoluted or lobulated nucleus.

(*21*) Megaloblast. An early type. The structure of the nuclear chromatin is fine and there are two nucleoli. The cytoplasm has an opaque greenish blue color, but shows a slight tendency to become acidophilic near the lower pole of the nucleus. The margins of the cell look thick.

(*22*) Megaloblast. A late type, with acidophilic cytoplasm and a nucleus which is becoming pyknotic and is undergoing a curious type of fragmentation.

(*23*) Megaloblast. An early type, with basophilic greenish cytoplasm. The nucleus has a relatively fine chromatin network, but somewhat coarser than in *21*.

(*24*) Immature normoblast, with faintly basophilic cytoplasm and characteristic wheel-spoke arrangement of the nuclear chromatin.

(*25*) Megaloblast. The nucleus is undergoing mitotic division. The cytoplasm shows fine basophilic stippling. These four megaloblasts were drawn from the blood of a patient with advanced pernicious anemia who had had no liver therapy.

(*26*) Reticulocyte, vitally stained with cresyl blue.

(*27*) Normoblast, with a pyknotic nucleus and fine basophilic stippling.

(*28*) Red cell, with a large nuclear fragment and fine "chromatin dust."

(*29*) Red cell, showing basophilic stippling and a Howell-Jolly body.

(*30*) Red cell, showing a Cabot ring. The cytoplasm is diffusely basophilic.

(*31*) Megakaryocyte, from a case of pernicious anemia. The cytoplasm is sky blue, with small clumps of bright red granules, identical in appearance with those of the platelets. The nucleus may be round or oval rather than elongated, as in this cell. Wilson's stain. (× 1200.)

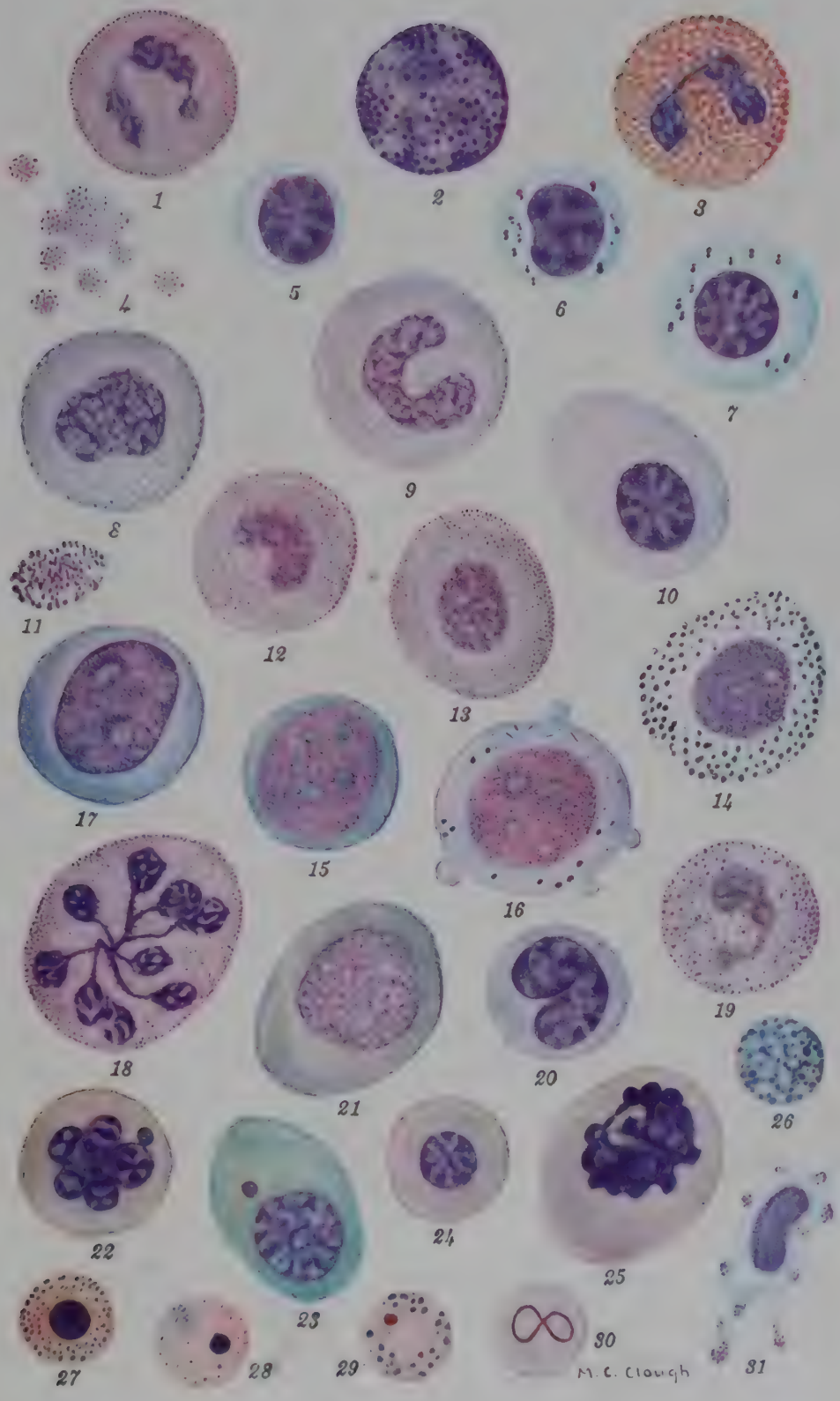


PLATE II

Types of normal and pathologic blood cells.
(For descriptive legend see p. 420.)

These cells are difficult to differentiate from lymphoblasts and other primitive leukocytes, and in some cases it is impossible to do so with the methods of study now available. Such undifferentiated cells are sometimes termed *stem cells* and are regarded by some as common ancestors of all the types of leukocytes.

In some cases small myeloblasts with dense, heavily staining nuclei occur, practically indistinguishable from lymphocytes. Presumptive evidence that questionable cells are myeloblasts may be obtained by demonstrating the occurrence with them of myelocytes (containing peroxidase-staining granules).

Lymphocytes. The lymphocytes in the blood of a normal adult are nearly all (mature) *small* cells, about the size of a red cell. The nucleus is relatively large, round or oval, eccentric, and often notched, or rarely, deeply lobed. It contains irregular, coarse, dense masses of chromatin which take a deep violet-blue stain. These masses are not sharply demarcated from the pale-staining parachromatin of the nucleus. There may be one or two nucleoli, visible only in crushed or flattened cells. The cytoplasm is sky blue or deep blue, but paler than the nucleus, and it usually shows a narrow crescentic pale zone next to the nucleus. No granules are present in fresh preparations, but in stained films about one-third of the cells show a few reddish-violet "azure" granules. Lymphocytes never show granules with the peroxidase stain.

Large lymphocytes differ chiefly in having more abundant cytoplasm. The nucleus is somewhat larger and usually takes a paler stain. The chromatin masses are often finer, and such cells are less mature than the normal small lymphocyte. These cells occur in small numbers in adults, and are common in children. There is little to be gained by separating large from small lymphocytes in the differential count.

Lymphoblasts, primitive lymphocytes, are large cells with abundant cytoplasm which usually is strongly basophilic. They often show a perinuclear pale zone, and may contain azure granules. The nucleus stains less intensely, and shows fine chromatin masses, although these are usually coarser than in the myeloblast. There are usually two or three nucleoli with a dense nucleolar membrane. The most primitive forms are practically indistinguishable from myeloblasts. Lymphoblasts occur chiefly in acute cases of lymphatic leukemia, but a few may be found in acute infections, or other conditions in which the production of lymphocytes is stimulated. The term *Rieder cell* has been applied to lymphoblasts with lobulated nuclei. Lymphocytes and lymphoblasts (in hanging-drop preparations) are motile (see p. 393).

Wiseman has described progressive changes in the lymphocytes which he regards as evidence of increasing maturity. He stresses the disappearance of nucleoli, decrease in number and disappearance of mitochondria, decrease in the degree of basophilia of the cytoplasm, and finally pyknosis of the nucleus and rupture of the cell membrane. Others question the reliability of basophilia as a criterion of immaturity in lymphocytes, since this does not always parallel those features of the nuclear structure (particularly fineness of the chromatin masses) which they regard as most significant.

Plasma Cells. Plasma cells are large cells with abundant, deeply basophilic cytoplasm which often contains azure granules, and may contain vacuoles. There is usually a pale perinuclear area. The nucleus is eccentric and contains coarse, dense masses of chromatin which tend to show a radiating, wheel-spoke arrangement. Immature plasma cells with nuclei of fine texture may occur in leukemia. Plasma cells have been regarded generally as lymphoid in origin, but they are not immature lymphocytes. Some believe them to be a distinct and independent type of cell, related to Türck's "irritation" cells. They are rare in normal blood, but a few may be present in any condition in which there is a lymphocytosis, especially in German measles, infectious mononucleosis, and in a few cases of multiple myeloma.

Türck's Irritation Cells. These are large cells with deeply basophilic cytoplasm, which is often vacuolated, but contains no granules. They were regarded by Naegeli as plasma cells, or pathologic lymphoblasts, because they have a lymphocytic type of nucleus. This

is usually finer in texture than that of the plasma cells. They may occur in any condition in which leukocyte formation is markedly stimulated.

Monocytes. Monocytes are large cells with abundant grayish-blue cytoplasm which is thickly studded with fine, pale-lilac granules, a little bluer than the neutrophil granules. In heavily stained films the granules in the older cells may be quite coarse and bluish. The margins of the cell are thin and often irregular. The nucleus in the older cells is kidney-shaped or horseshoe-shaped, and often shows small, irregular lobulations along the concave margin (*transitionals* of Ehrlich). The younger forms have round or oval nuclei (*large mononuclears* of Ehrlich). The surface of the nucleus often shows several deep creases which give it a coarsely convoluted appearance. The nucleus takes a pale bluish-violet stain, and contains a characteristically reticulated chromatin network with slight thickenings at the intersections of the threads. In the younger cells it has a fine, spongy texture which may be compared with that of fine lace, whereas in the older cells it is coarser and stains more intensely. There are no nucleoli. Monocytes show ameboid motility and are actively phagocytic (see p. 394).

Monoblasts, the primitive monocytes, differ in having a more basophilic cytoplasm and sparse granulations. The nucleus is fine in texture and may contain a few small, pale-blue nucleoli.

Pathologic Leukocytes. Pathologic leukocytes are cells which, in some respect, differ markedly in their appearance from normal cells of their type. The difference may result from a *developmental anomaly* in conditions in which blood-cell formation is disturbed. There may be an abnormality in the size of the cell, in the quantity or staining properties of the cytoplasm or its granules, or in the size, the staining, or the degree of segmentation of the nucleus. Pathologic lymphocytes occur frequently in infections, and do not necessarily suggest leukemia.

Pathologic changes may also occur as a result of *toxic injury* to the cells in acute infections. Such cells may show vacuoles in the cytoplasm or the nucleus. The margins of the cell may be irregular or "moth eaten." The neutrophilic granules may be sparse or absent, or may stain abnormally, and fail to take the peroxidase stain. The presence of coarse bluish granules has been emphasized and also bluish staining areas in the cytoplasm. The nucleus may be swollen and show loss of its finer structure, or it may be pyknotic. If marked in degree, such changes have a bad prognostic significance.

Smudged cells, if not due to poor technic in making the film, suggest the presence of large, fragile cells. In certain cases of leukemia these may be numerous, and have been termed "basket" cells. Even in normal blood many of the neutrophils at times may be smudged. Sabin believes that these smudges represent showers of disintegrating leukocytes, because she finds numerous nonmotile leukocytes in supravitally stained films made at the same time.

Origin and Life of Blood Cells

The primitive precursors of the blood cells are the cells of the *reticulo-endothelial system.* These cells are distiguished physiologically by their capacity to engulf and store particulate matter, such as India ink, or fragments of red cells; or colloidal dyes, such as pyrrol blue. These cells comprise: (1) *reticulum cells,* irregularly stellate in shape, scattered throughout the body in the connective tissue about the blood vessels. According to Corner these cells can be distinguished by demonstrating the presence of reticulum fibrils within the substance of the cell by special staining methods (disputed). (2) The *specialized endothelial* cells of the capillaries and sinuses of certain limited regions of the body: the bone marrow, spleen, liver, lymph follicles, hypophysis, adrenal cortex. (3) The *macrophages,* also known as wandering cells, clasmatocytes, histiocytes, and endotheliocytes (and probably the monocytes), which are derived from either or both of the preceding types of cells.

The *primitive red cells* are derived from vascular endothelium. In the early embryo the process of red-cell production is widely distributed, but it soon becomes largely concentrated in the liver, and later in the spleen. At birth it is largely confined to the red bone marrow, all bones participating. After about the sixteenth year it has disappeared from the long bones except small foci in the proximal ends of the femur and humerus, and in the normal adult it is largely confined to the vertebrae, ribs, sternum, skull, and pelvis. In response to the stimulus of an anemia, red-cell formation is often resumed in the long bones, occasionally in the spleen, and rarely even in the liver.

According to Doan, in the adult the red cells are normally produced by intravascular proliferation of the endothelium of intersinusoidal capillaries in the marrow. These capillaries are believed usually to be shut off (functionally) from the general circulation but open occasionally to discharge red cells as these mature. The bulk of the erythropoietic tissue is made up of the cells described as "immature normoblasts." Some of these continue in a state of active proliferation, whereas others develop by a process of maturation into mature red cells. In certain severe anemias, particularly in pernicious anemia, these cells are replaced by more primitive types of erythroblasts, the macroblasts or megaloblasts.

The ultimate *ancestors of the granular leukocytes* are believed to be primitive reticulum cells. In the embryo the leukopoietic tissue has much the same distribution as noted in the case of the red cells. In the normal adult their formation is largely limited to the marrow of the flat bones. When their proliferation is strongly stimulated, as in leukemia, the distribution becomes widespread, involving not only the spleen and liver, but also the lymph nodes, and sometimes the skin and the connective tissue of the kidneys and other organs, particularly about the blood vessels. Normally the proliferating tissue is largely composed of differentiated myelocytes, but in leukemia these may be replaced by primitive myelocytes or myeloblasts.

The *lymphocytes* are formed in the lymph follicles, wherever these may be located. Their immediate precursors are the large cells in the center of the follicles. Their more primitive precursors, the lymphoblasts, are probably derived from reticulum cells.

The *monocytes*, according to Sabin and many others, are derived from reticulum cells, largely but not exclusively in the spleen. Naegeli and some others believe that they arise in the bone marrow, from myeloblasts or closely related cells. The consensus now is that they constitute a third type of leukocytes, distinct from both the lymphocytes and the granulocytes. The exact relationship of the monocytes to the *histiocytes*, or *clasmatocytes*, is still in dispute. Sabin regards the latter as a distinct type of cell derived from endothelium. There is strong evidence that under special conditions, at least, histiocytes are also derived from monocytes.

There is still disagreement as to many of the details of the process of blood-cell formation, particularly as to the relation of the primitive types of cells to one another. There is much to support the view that even in the adult there are primitive (reticulum) "stem" cells which under certain conditions may give rise to any one of the three main types of leukocytes. In leukemias of any type there is widespread proliferation of the corresponding type of cell, which may be found practically wherever reticulum cells occur. On the other hand there is no convincing evidence of the transformation of one type of leukocyte into another after definite differentiation has occurred. Table 46 summarizes one of the more widely accepted theories as to these relationships.

The leukocytes perform their functions mainly in the tissues and not in the blood. They utilize the blood stream only as a means of transportation. Their average life, or at least their stay in the blood, is short, probably from a few hours to four days at most. The normal stimulus to their production is a chemical one, probably nucleic acid or other products liberated from the nuclei of disintegrating leukocytes. That enormous numbers are formed daily may be surmised from the fact that most of the red marrow (five-sixths according to some estimates) is occupied with their formation. Some of the leukocytes

Table 46

ORIGIN OF THE BLOOD CELLS

Maturation Leads	*Reticulo-endothelial System*				
	Extravascular Origin			*Intravascular Origin*	*Intra- and Extravascular Origin*
Primitive Fixed Cells	*Reticulum Cell (Multipotential)*			*Endothelial Cell (Duopotential)*	
Primitive free cells	Primitive cell (multipotential)				
Differentiating mechanism, environment or specific stimulus	In bone marrow	In connective tissue, spleen, lymph nodes	In lymphatic tissues	In intersinusoidal capillaries of bone marrow (Doan)	All other endothelium
Unipotential blood cells	Myeloblast	Monoblast	Lymphoblast	Megaloblast Erythroblast	Clasmatocyte (histiocyte)
Young cells	Myelocyte	Premonocyte	Young lymphocyte	Normoblast	(Maturation not described)
Mature cells	Poly	Monocyte	Mature lymphocyte	Reticulocyte Red cell	
Degenerating cells	Nonmotile (Sabin)	?	Old lymphocyte	Fragmented red cells	

This table summarizes the views of those hematologists who believe in the independent origin of each of the types of blood cells ("polyphyletic school"). Those belonging to the "unitarian school" believe that the common ancestor of the different types of cells ("primitive cell" in this table) is a lymphocyte and that transformation of one type of cell into another may occur, even up to the mature stage. (After Wiseman, *Journal of the American Medical Association,* Nov. 17, 1934.)

disintegrate in the tissues and are removed by the macrophages. Many leave the body by migrating through the mucous membranes of the alimentary tract.

Under normal conditions the average life of the red cells is probably about 12 weeks (estimates vary from 2 to 12 weeks). Hence the cells from about 50 ml. of blood, about 250 billion in number and containing about 8 Gm. hemoglobin, are removed and replaced daily. Red cells are not normally hemolyzed in the circulation. It is probable (Rous and Robertson) that, as a result of the buffetings the cells receive in the circulation, they are gradually broken to pieces without loss of hemoglobin to the plasma. The fragments are ingested by the phagocytic cells of the reticulo-endothelial system, largely, but not exclusively, in the spleen. The iron is split off and stored in the liver and spleen. The balance of the hemoglobin molecule is split up, and part is converted into bilirubin in these cells, which secrete it into the blood. It is then taken up by the glandular cells of the liver, and largely excreted in the bile. The bilirubin is converted into urobilinogen in the intestine and eliminated in the feces. A part of the bile pigments is reabsorbed from the intestine. This is not utilized but is largely re-excreted by the liver, although normally small amounts of urobilinogen are excreted in the urine.

Normal Differential Count

The average figures for the normal adult are given in the following table.

Table 47

NORMAL DIFFERENTIAL COUNT

	In percentage		*Absolute Number per Cu. mm.*	
	Average, %	*Usual Range, %*	*Average*	*Usual Range*
Neutrophils: total	66	50 to 70	4500	3000 to 6000
Segmented nuclei	62	50 to 65	4200	3000 to 6000
Nonsegmented nuclei	4	2 to 8	300	200 to 700
Metamyelocytes	0	0 to 0.1	..	..
Eosinophils	1.5	0.5 to 3	100	50 to 300
Basophils	0.5	0 to 0.75	25	0 to 75
Lymphocytes: total	26	20 to 30	1800	1000 to 3000
Large	1	0 to 6	100	0 to 600
Small	25	20 to 30	1800	1000 to 3000
Monocytes	6	3 to 8	450	300 to 600
Total leukocyte count	..	..	7000	5000 to 10,000

In infants after the first few days the lymphocytes constitute 40 to 60 per cent of the total count, and the neutrophils are correspondingly reduced (30 to 50 per cent). The average adult formula is usually attained at about five years of age, but this is quite variable (2 to 10 years or more).

Leukocytosis

The term "leukocytosis" signifies an increase in the total leukocyte count (above 10,000). Since in most cases this is due to an increase in the neutrophils, it is often used inexactly to indicate a *neutrophilic leukocytosis.* Such a leukocytosis is called *relative* if the total leukocyte count is within normal limits, but the percentage of neutrophils is increased (above 70 per cent). It is *absolute* if the total number is increased, as well as the relative number. A relative neutrophilic leukocytosis usually has about the same significance as a slight absolute leukocytosis.

Occurrence. *Normal individuals* may show a neutrophilic leukocytosis:

1. After muscular activity (sometimes marked) and convulsions.
2. After cold baths (a rapid redistribution of cells in the circulation).
3. A digestive leukocytosis has often been described, but recent work has created grave doubt as to its occurrence.
4. In infants during the first few days.
5. In pregnancy during the last few weeks (slight), and in the puerperium (sometimes marked, up to 20,000).

It occurs in the following *pathologic conditions:*

1. In most *acute infections;* particularly acute pyogenic infections, intra-abdominal inflammations, general sepsis, pyelonephritis, tonsillitis, scarlet fever, acute rheumatic fever, pneumonia of all types, and meningitis. The total leukocyte count in these conditions usually ranges from 12,000 to 20,000; in lobar pneumonia it often reaches 30,000 to 40,000. It may rarely rise to 100,000.

2. In *intoxications;* as with illuminating gas, lead (up to 20,000 in lead colic), after such drugs as turpentine, acetanilid, potassium chlorate, phenyl hydrazine, digitalis; with venom of arthropods; in acute gout; in diabetic acidosis; and in uremia.
3. After acute hemorrhage and acute hemolysis of red corpuscles.
4. Malignant neoplasms, especially rapidly growing tumors of the liver, gastrointestinal tract, and bone marrow (metastatic).
5. After operations, for 12 to 36 hours, particularly if there has been much tissue injury.
6. Necrosis of tissue as in coronary thrombosis, and after burns.
7. Myelogenous leukemia.

Significance. In general, a leukocytosis may be regarded as a response of the bone marrow to an increased demand for leukocytes. The degree and type of response vary (1) with the intensity of the stimulus (which depends largely on the species of the infecting organism, on its virulence, and on the location and extent of the area involved), and (2) on the reacting power of the individual. A mild infection may call forth an insignificant response. If the infection is overwhelming, or if the reacting power of the marrow is seriously impaired, there may be no leukocytosis, but even a leukopenia. In an individual with adequate reacting power the degree of leukocytosis is very roughly proportional to the severity of the infection. In interpreting the leukocytic response to an infection, however, changes in the differential count and qualitative alterations in the leukocytes are more significant than an increase in their total number.

A great deal of work has been devoted to this problem, in an attempt to find some characteristic change which would have prognostic significance in infections, and which might serve as an indication for immediate operation in such conditions as acute appendicitis. The earlier studies emphasized the significance of a *rise in the percentage of neutrophils* and the relation of this rise to the increase in the total leukocyte count. Thus Sondern regarded the latter as a measure of the resistance of the individual, whereas the degree of the increase in the percentage of neutrophils measured the severity of the infection or the "degree of toxic absorption." Wilson devised a formula to express this numerically.

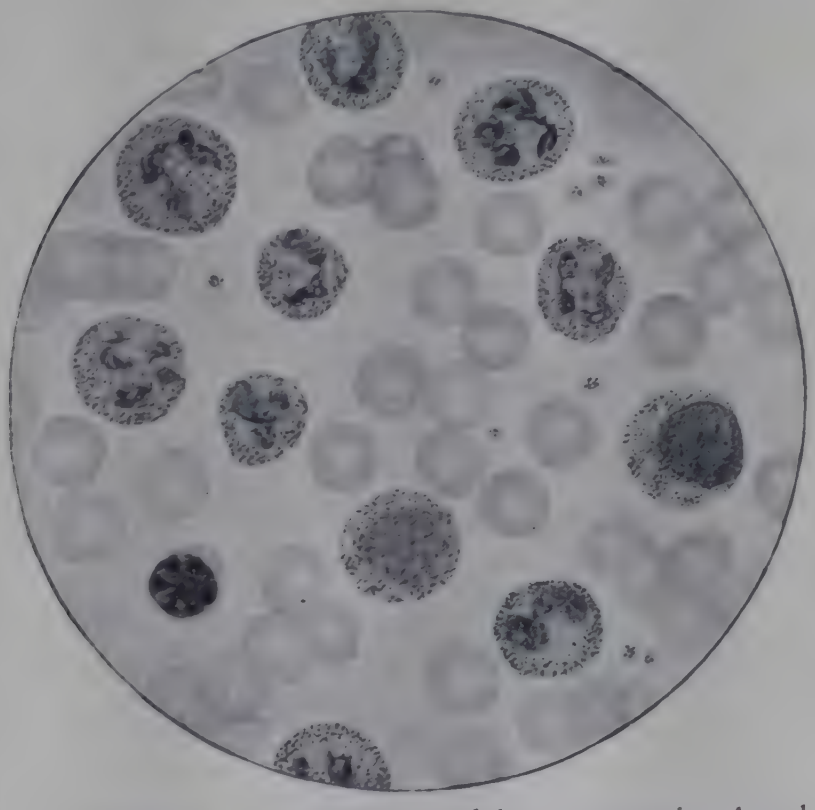
$$\textit{Index of resistance} = (T - 10) - (P - 70).$$

T = the total leukocyte count in thousands.
P = percentage of neutrophils.
A negative index indicates a low resistance and a poor prognosis.

In general, if a blood-stream invasion or a specific acute infection (like pneumonia) can be excluded, a neutrophil percentage of 85 or over points to pus formation or gangrene whereas a percentage under 80 is against this. Individual exceptions occur, however, particularly in children and in patients with such infections as typhoid fever or tuberculosis.

More reliable information is obtained by a study of the neutrophils from the standpoint of their *maturity*. The available reserve supply of preformed leukocytes is relatively small, and an increased demand must be met largely by the production of new cells by the bone marrow. When hastily produced in response to an infection, many of the cells are hurried into the circulation before development is completed. Arneth was the first to emphasize the significance of this phenomenon, and he attempted to measure it quantitatively by utilizing the degree of segmentation of the nucleus as a criterion of maturity. In making differential counts he divided the neutrophils into five groups. In the first group he included all cells with unsegmented nuclei, and in the second to the fifth groups (inclusive) he put cells with nuclei composed of two to five lobes respectively. He

gave the following figures as the average distribution in normal blood: 5 per cent, 35 per cent, 41 per cent, 17 per cent, 2 per cent. He calculated a *numerical "index"* by adding together the percentage of cells in the first two groups, and half the figure for the third group (normal index 60). In cases of active infection the relative number of cells in the first two groups was increased, and as these happened to be recorded on the left hand side of his tally sheets, this change was termed a *"shift to the left."* He regarded this shift as far more significant than a rise in the total leukocyte count. However, grave objections, both theoretical and practical, have been raised to Arneth's counts, and they have been practically abandoned as a clinical procedure.



Leukocytosis (55,000). Acute lobar pneumonia. Actual field. Marked regenerative "shift to the left." Two myelocytes in field. (*Lower right*) Cell showing toxic-degenerative changes in the granules.

Several other investigators have suggested simplified procedures to estimate the degree of immaturity of the leukocytes. Of these we shall give only that of Schilling, which is the one most frequently employed. This is best understood by a study of the following illustrative counts, selected from his book. He subdivides the neutrophils into four classes, which have already been described. Good, thin films are indispensable for these counts. The differentiation of segmented from nonsegmented nuclei is in part a subjective matter, and the examiner should establish his own standard of normal by counts on normal blood.

As a rule in acute infections, particularly those which are accompanied by a neutrophilic leukocytosis, there is an increase in the percentage of cells with nonsegmented nuclei (a shift to the left), which is roughly proportional to the severity of the infection.

Schilling distinguishes a "regenerative shift," in which "Jugendformen" (metamyelocytes) appear in the blood, from a "degenerative shift," in which the nonsegmented cells are "Stabzellen," with coarse, densely stained chromatin. The

Table 48

ILLUSTRATIVE DIFFERENTIAL COUNTS BY SCHILLING'S METHOD, COMPILED FROM SCHILLING'S "THE BLOOD PICTURE"

	Basophils	*Eosinophils*	*Myelocytes*	*Metamyelocytes (Jugendformen)*	*Nonsegmented Neutrophils (Stabzellen)*	*Segmented Neutrophils*	*Lymphocytes*	*Monocytes*	*Total Leukocyte Count*
Normal	1	2	0	0	4	63	23	6	6,000
Normal Range	0–1	2–4	0	0–0.1	3–5	51–67	21–35	4–8	5,000–8,000
Appendicitis (mild)	0	1	0	0	12	63	16	8	10,000
Appendicitis:									
(1) Wound infection	1	3	0	2	9	65	9	11	13,000
(2) Peritonitis	0	0	2	21	29	41	2	5	33,000
(3) Agonal	0	0	12	29	38	21	0	0	25,000
Pneumonia (favorable)	0	0	0	4	32	51	12	1	12,000
Pneumonia (senile, fatal)	0	0	1	30	18	35	11	5	8,600
Bacterial endocarditis	0	2	0	2	20	53	17	6	5,000
Tuberculosis									
(1) Mild, active	1	2	0	0	3	39	39	16	Low
(2) Advanced	0	0	0	6	39	17	13	25	High
Malaria (MT) paroxysm	0	0	0	22	28	20	20	10	High
Malaria (BT) afebrile period	0	2	0	7	14	46	15	16	6,000
Typhoid fever	0	0	0	0	30	20	38	12	3,000

latter are probably to be regarded as the result of pathologic maturation, rather than an indication of simple immaturity. A regenerative shift is the more significant.

In general, the following points indicate a severe infection and usually have an *unfavorable prognostic significance:* (1) An extremely high total leukocyte count with a high percentage of neutrophils. (2) The failure to develop a leukocytosis, or a leukopenia. (3) A high percentage of immature cells, especially if they outnumber the mature cells. (4) Absence of eosinophils. (5) A marked reduction in the absolute number of lymphocytes. (6) Many cells showing toxic-degenerative changes in the nucleus or cytoplasm. Conversely a favorable significance may be attached to the absence or progressive abatement of these characteristics. Although caution must be used in interpreting the findings in any individual case, such a study undoubtedly gives information of great practical value. A definite trend in either direction as shown by successive examinations is much more significant than the result of a single examination.

Eosinophilia. This occurs chiefly in three groups of conditions: (1) *Infection with animal parasites.* In *trichinosis* it is nearly constant and usually marked (up to 70 per cent of 35,000 cells), but it may be absent in severe acute cases. It usually occurs in echinococcus and hookworm infections of significant degree, in the early stages of schistosomiasis, and less regularly with ascaris, taenia, whipworm, strongyloides, etc. In Guam among the natives it is difficult to find an eosinophil count of less than 15 per cent. It is rare in amebiasis. (2) *Skin diseases* of any type, depending more upon the extent than upon the type of skin involvement. (3) *Allergic reactions* to foreign protein, including bronchial asthma, hay fever, urticaria, angioneurotic edema, mucous colitis, and serum disease. It

also occurs in (4) chronic myelogenous leukemia; (5) the acute stage of scarlet fever and in chorea; and (6) the stage of recovery from many acute infections (in slight degree). It is seen occasionally (7) in Hodgkin's disease (up to 60 per cent in rare instances), (8) with certain tumors of the ovary, (9) in focal lesions of the bone marrow, (10) after splenectomy, (11) in pernicious anemia after liver therapy, particularly following ingestion of raw liver (up to 20 per cent, rarely even to 60 per cent), (12) in periarteritis nodosa (10 per cent of cases), and (13) in Loeffler's syndrome and tropical eosinophilia.

Eosinophils are diminished in the acute phase of most severe infections.

Basophilic Leukocytosis. A basophilic leukocytosis occurs commonly in chronic myeloid leukemia. In other conditions it is infrequent and of no known diagnostic significance.

Lymphocytosis. A relative lymphocytosis occurs in most of the conditions which show a leukopenia.

An *absolute lymphocytosis* occurs in certain infections: (1) Whooping cough, including the secondary bronchopneumonias which follow it. A lymphocytosis is nearly constant, and usually marked, from 20,000 to 40,000, rarely to 100,000, with 60 to 80 per cent of normal small lymphocytes. (2) Infectious mononucleosis (glandular fever). (3) Less regularly and less markedly in German measles (with plasma cells), Brucella infection, and mumps. (4) In some cases of tuberculosis, chiefly mild or moderately active cases running a favorable course. (5) In young children, occasionally, in infections which usually cause a neutrophilic leukocytosis. (6) During convalescence from any acute infection. (7) In hyperthyroidism. (8) After exposure to ultraviolet radiation. (9) In lymphatic leukemia. (10) In acute infectious lymphocytosis.

Monocytosis. This occurs: (1) In many cases of malaria and other *protozoan infections,* including trypanosomiasis, kala-azar, and amebiasis. (2) Less regularly and often only in slight degree in variola, typhus, spotted fever, dengue, yellow fever, measles, and syphilis. (3) In bacterial endocarditis (capillary blood). (4) In *active progressive tuberculosis.* A reversal of the usual lymphocyte-monocyte ratio suggests a dissemination of tubercles and has an unfavorable prognostic significance. (5) In Hodgkin's disease. (6) In Banti's disease. (7) In poisoning with tetrachlorethane. (8) In monocytic leukemia.

A slight increase in monocytes occurs frequently at the height of many acute infections.

Myelocytosis. Myelocytosis, or the presence of myelocytes in the circulating blood, occurs chiefly (1) in myelogenous leukemia. In usually small numbers myelocytes may appear (2) in any condition associated with a hyperleukocytosis, especially in children. (3) In severe infections or intoxications, regardless of the total leukocyte count, in which there is a marked "shift to the left" (a "leukemoid reaction"). In marked reactions myeloblasts may also be found. (4) In pernicious anemia and other severe anemias. (5) In focal lesions of the bone marrow, especially metastatic neoplasm.

Leukopenia

A notable reduction in the leukocyte count below 5000 must be mainly at the expense of the neutrophils. It occurs: (1) Regularly in certain infections, if uncomplicated: typhoid fever, measles, influenza, dengue, kala-azar, and malaria (except during the paroxysms); occasionally in the initial stages of smallpox, and in some cases of tuberculosis, especially extensive glandular tuberculosis. (2) Overwhelming infections which are usually associated with a leukocytosis. (3) Malignant neutropenia (agranulocytic angina). (4) Acute or chronic poisoning with such drugs as benzol, acetanilid, the sulfonamides, aminopyrine, lead, mercury, and arsenic (arsphenamine), thiouracil, dinitrophenol, barbiturates (rarely). (5) During acute anaphylactic reactions. (6) After excessive radiation. (7) Cachectic or debilitated states. (8) "Exhaustion" of the marrow, as in idiopathic aplastic anemia, and some cases of leukemia. (9) Displacement of the leukopoietic tissue of the marrow by other tissue, as in pernicious anemia, leukemia, or metastatic neoplasm. (10) Primary splenic neutropenia. (11) With a deficiency of folic acid.

A "simple leukopenia," usually of moderate degree (3000 to 5000), is observed occa-

sionally in otherwise apparently normal individuals The differential count may be normal, or there may be a slight relative lymphocytosis. There is not necessarily any evident decrease in resistance to ordinary infections.

The *lymphocytes* are *reduced* in absolute number (1) after excessive radiation, (2) during the acute stage of most infections, and (3) in conditions in which the lymphoid tissue is replaced by other tissue, as in myelogenous leukemia, advanced Hodgkin's disease, and extensive tuberculosis or carcinomatosis of the lymph nodes.

Blood Platelets

The blood platelets, or thrombocytes, are round or oval bodies usually 2 to 3μ in diameter, although they vary greatly in size and shape. In films they show a marked tendency to form clumps. With Wright's stain they show a faintly bluish background studded with numerous fine, dull purplish-red granules which resemble chromatin. They contain no nucleus. They are believed to arise from the megakaryocytes, the multinucleated giant cells of the bone marrow, as pinched-off fragments of the cytoplasm of these cells. Howell (1937) has demonstrated that in cats they are formed from megakaryocytes lying in the pulmonary capillaries rather than in the bone marrow. In pathologic conditions, particularly in chronic myelogenous leukemia, occasionally in Hodgkin's disease and pernicious anemia, large masses of cytoplasm may be found in blood films.

In these conditions *megakaryocyte nuclei* are occasionally found in the blood. The typical, large lobate nuclei seen in marrow films cannot pass through the pulmonary capillaries, but small nuclei or nuclear fragments may get through. They are two to four times the diameter of a red cell or larger, round, oval, or irregular in shape, and take an intense, reddish-purple stain. The chromatin tends to be condensed slightly in coarse splotches or irregular bands separated by somewhat paler staining areas, giving the nucleus an irregularly striped or coarsely spotty appearance. The nuclei may be naked, or there may be small, ragged fringes of platelet material attached.

Platelets are indispensable for normal blood coagulation. Their life is brief, about three or four days. They are thought to be removed by the spleen and other reticuloendothelial tissues. The stated normal number varies with the method used to count them (see p. 386).

Platelets are increased in most conditions associated with active cell formation in the marrow. Among these are: (1) many acute infections, (2) after hemorrhage, (3) chronic myelogenous leukemia, (4) polycythemia, (5) Hodgkin's disease.

Platelets are diminished in: (1) idiopathic purpura hemorrhagica, (2) pernicious anemia, (3) aplastic anemias, (4) acute leukemias (the "primary" thrombocytopenias), (5) some severe infections, (6) acute or chronic poisoning with benzol, radium, arsphenamine, gold salts, nirvanol, sedormid, barbiturates (rarely), etc., and (7) after excessive radiation (secondary thrombocytopenias).

CHAPTER 15

Diseases of the Blood

Diseases Involving Primarily the Red Cells

Anemias

By *anemia* is meant a reduction below the normal in the amount of hemoglobin (and usually in the number and volume of the red cells) per unit volume of blood. It indicates a failure of the red-cell-forming tissues of the bone marrow to keep pace with the loss or destruction of red blood cells. It is a symptom of a great variety of unrelated diseases.

There is no single scheme of *classification* of the anemias which is comprehensive and satisfactory. The most logical method is based on the *pathogenesis* of the anemia. (1) Those due to loss of blood, the posthemorrhagic anemias. (2) Those due to inadequate blood formation, the deficiency anemias and the aplastic anemias. (3) Those due to increased blood destruction, the hemolytic anemias. There are practical difficulties in the application of this classification, because in many cases inadequate blood formation and increased blood destruction both play an important role. In general a low reticulocyte count suggests inadequate blood formation, and an increase in bilirubin in the serum, increased blood destruction.

From the *morphologic* standpoint the anemias may be classified according to the mean corpuscular hemoglobin or color index as hyperchromic, normochromic, or hypochromic; and according to the mean corpuscular volume or volume index as macrocytic (megalocytic), normocytic, or microcytic. This classification has practical significance, since, in general, macrocytic anemias are benefited by liver or liver extract, whereas the hypochromic anemias are helped by iron. The terms, however, should be regarded as descriptive, and not as diagnostic. The terms "normocytic" and "normochromic" should be understood to mean merely that the *average* volume or hemoglobin content of the red cells is within normal limits. Otherwise they may be misleading, since the cells may be abnormal in other respects, and individual cells may be markedly abnormal in size and color.

In a strict sense there are no hyperchromic anemias, since normally the red corpuscles are saturated with hemoglobin and supersaturation has not been observed. The term hyperchromic, however, is sometimes applied in a descriptive sense to those anemias in which the red cells in films appear unusually dark because of an increase in the thickness of the cells.

The current tendency is to drop the use of the old terms "primary anemia," supposedly due to some inherent disease of the blood-forming tissues; and "secondary anemia," due to some obvious extraneous cause, although it is sometimes convenient to use them.

Anemias due to loss of blood

These anemias are hypochromic and usually microcytic.

Acute Posthemorrhagic Anemia. The danger to life from a single profuse hemorrhage is from circulatory failure (shock) due to the lack of a sufficient volume of blood to fill the vessels, and not from a deficiency of hemoglobin. The

first step in regeneration after an acute hemorrhage is the restoration of plasma volume, by the passage of tissue fluids into the vessels. This results in a dilution of the blood, with a gradual fall in red-cell count and hemoglobin, which is not complete until from 24 hours to three or four days after bleeding has ceased. The resulting anemia stimulates a rapid production and outpouring of new red cells which at first are normal reticulocytes. After a few days, however, the new cells tend to be imperfectly formed, to be inadequately supplied with hemoglobin, and to be hurried into the circulation before development (maturation) is complete. Reticulocytes are increased, polychromatophilic cells appear, and occasionally a few normoblasts. There is usually a neutrophilic leukocytosis and an increase in platelets. The new red cells tend to be smaller than normal and are pale. The color index and volume index fall. With the influx of new cells there is a gradual rise in the red-cell count and (more gradual) in hemoglobin. The maximum abnormalities in the cells, however, are not reached until about the sixth or eighth day. In otherwise normal individuals completely normal conditions are restored usually after about 30 to 60 days. As a rule these changes in the red cells are only slight in degree after a single hemorrhage, no matter how profuse. They may become marked in the case of continuing or quickly repeated bleeding.

Chronic Posthemorrhagic Anemia. The changes in the blood are usually similar to those described above, but they tend to become more marked. In severe cases there is a marked degree of anisocytosis, a majority of the red cells are smaller than normal in diameter and volume, microcytes are numerous, and some poikilocytes may be present. As the iron stores of the body become depleted the concentration of hemoglobin in the cells diminishes, the color index and the saturation index fall, and the cells are pale. The centers may be colorless, so that the cells look like rings (pessary forms). There is no increase in the bilirubin in the serum. This is the typical picture of a *hypochromic microcytic anemia.* The degree of anemia may be severe. Counts of two million red cells and 20 per cent of hemoglobin are not unusual, and rarely they may fall to half these figures.

The bone marrow is hyperplastic, and the predominant cells are normoblasts. The fatty marrow of the long bones is often replaced by such red hyperplastic marrow.

While active formation of red cells continues, immature cells will be present. In patients with protracted bleeding, however, the marrow may become exhausted (aplastic). In such cases the immature red cells disappear, and there may be a reduction in the number of leukocytes and platelets. In exceptional cases of this type, particularly in patients having long-continued small hemorrhages, the few cells which are formed may be more nearly normal, and the color index may approach 1.0.

Identical changes are met with in *hookworm infection.* (See p. 608.)

ANEMIAS DUE TO INADEQUATE BLOOD FORMATION

Anemias Related to a Deficiency of Iron

These anemias are hypochromic, and usually microcytic in type. They include: (1) Anemias due to *lack of iron* in the diet, seen most frequently in infants and young children on a diet consisting largely or exclusively of milk. The anemia appears earlier and is more severe in children of anemic mothers, because of in-

adequate storage of iron in the fetal tissues. (2) Anemias due to *faulty absorption of iron* from the digestive tract, as in chronic diarrhea, colitis, and some cases of sprue and idiopathic steatorrhea. (3) Anemias due to *loss of iron* by external hemorrhage (already discussed). (4) Anemia in severe hookworm infection. (5) Anemia in some cases of cancer, particularly of the gastrointestinal tract. (6) Anemia in some patients after extensive operations on the stomach. (7) Idiopathic hypochromic anemia. (8) Chlorosis.

Idiopathic Hypochromic Anemia (Primary Microcytic Anemia, Simple Achlorhydric Anemia, Chronic Chlorosis). This is a chronic disease largely (95 per cent) limited to women, chiefly those between 20 and 50 years of age. Clinically it is characterized by an insidious onset, by the gradual development of marked weakness, lethargy, and nervous instability; and by digestive discomforts: gaseous distention, epigastric pain, occasionally diarrhea and anorexia, or a fickle appetite. This often leads the patients to avoid meats, fruits, and green vegetables—foods rich in iron—and thus the anemia is aggravated. Marked loss of weight is exceptional.

Soreness of the tongue and mouth is common. There is a *glossitis* and stomatitis which lead to atrophy of the mucous membrane. In at least half the cases there is atrophy of the papillae about the tip and margins of the tongue. The process often extends to the dorsum of the tongue, which becomes smooth and polished in appearance; and to the lips, which may show cracks and fissures about the corners of the mouth. In a small group of cases (*Plummer-Vinson syndrome, anemia with dysphagia*) it extends into the pharynx and hypopharynx, causing dysphagia, which is attributed to reflex spasm of the inferior constrictor.

In about half the cases the nails become tender, thin, and brittle; they tend to loosen from the nail bed, and may become flattened or even concave and spoon-shaped on the dorsal surface (*koilonychia*). Vaughan has observed this also in chronic posthemorrhagic anemia.

Paresthesias of the extremities are common, as in pernicious anemia, but combined sclerosis of the cord does not occur.

Menorrhagia is a common symptom, and the anemia may erroneously be attributed simply to the loss of blood. Otherwise there is rarely any abnormal tendency to bleed, or any disturbance of coagulation. Fertility is but little affected.

The *spleen* is enlarged in about 40 per cent of the cases.

The *skin* becomes inelastic and wrinkled; it may be waxy white or show slight brownish pigmentation. The sclerae are bluish white. There is never jaundice.

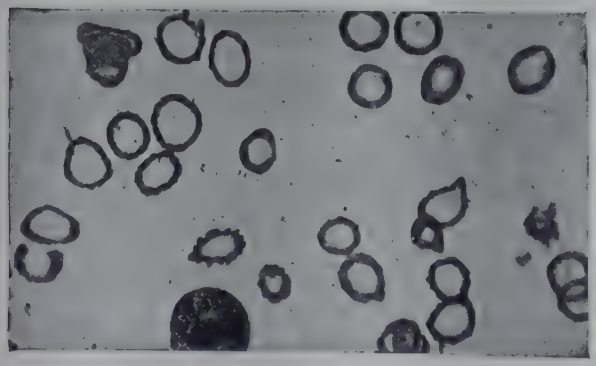
The *gastric juice* shows a hypochlorhydria or an achlorhydria in at least 85 per cent of the cases, and a complete achlorhydria after histamin in about 60 per cent. Mucus is abundant. The ordinary ferments are often diminished or absent, but the intrinsic factor of Castle is retained.

The *blood* shows all the features characteristic of a hypochromic microcytic anemia, as described in posthemorrhagic anemia. In the average patient the red cell count is between 3.5 and 4.0 million, the hemoglobin 6 to 8 Gm., or 40 to 50 per cent. In severe cases, they may fall to 1.5 million and 2.0 Gm. The striking features are the extreme pallor of the cells and the degree of reduction in the color index (0.3) and in the mean hemoglobin content (11$\gamma\gamma$) and hemoglobin concen-

tration (22 per cent) in the red cells which may develop. The leukocytes and platelets are usually normal.

In untreated cases reticulocytes are sparse. However, biopsy shows the bone marrow to be markedly hyperplastic (normoblastic). There appears to be some obstacle to the maturation of the cells. An effective stimulus to their maturation and delivery into the circulation is provided by the administration (by mouth) of large doses of iron (6 Gm. or more per day of iron and ammonium citrate, or 1.5 Gm. of ferrous sulfate). In severe cases within three or four days after an adequate dose of iron is started, there is a *rise in reticulocytes* which reaches a peak on the seventh or eighth day, and which is roughly inversely proportional to the hemoglobin percentage. A satisfactory response is indicated by a rise in reticulocytes to 15 per cent if the hemoglobin is 20 per cent; to 8 per cent, if the hemoglobin is 40 per cent; and to 5 per cent, if the hemoglobin is 60 per cent; and by an average daily rise in hemoglobin of at least 1 per cent (in some cases 2 per cent). An equally striking rise may be obtained in severe hypochromic anemia of other types, as in hookworm anemia (Castle and Rhodes, 1932), and even (temporarily) in cancer of the stomach. There is prompt relief (not always complete) of the symptoms and subsidence of the physical signs, except that the lingual atrophy and the achlorhydria usually persist. Liver extracts are ineffective.

The disease is rarely directly fatal, even if untreated, but it often causes a protracted and profound degree of chronic invalidism. There is little tendency to spontaneous remission until after the menopause. An exacerbation may be precipitated by a pregnancy, or by intercurrent infections.



Red blood corpuscles showing deficient hemoglobin (achromia). From a well marked case of chlorosis. Wright's stain. (× 750.) (Courtesy, J. C. Todd: Manual of Clinical Diagnosis, Philadelphia, W. B. Saunders Co.)

The *etiology* is still obscure. A major factor is deficient iron absorption resulting from the defective gastric secretion. In some cases the latter is attributable to an inherent constitutional (sometimes familial) defect. In some it may be due to a chronic gastritis. A severe anemia of any type, however, may cause a temporary disappearance of free hydrochloric acid from the gastric juice. An identical condition has been described in patients (male as well as female) after gastroenterostomy, after extensive gastric resection, and in some cases of cancer of the stomach. Other contributing causes are an inadequate, ill-balanced diet, and the drain on the iron reserves resulting from menstruation and pregnancy. The defect is permanent, since relapse occurs if the administration of iron is stopped.

The great significance of *chronic occult bleeding* has been emphasized recently, notably by Heath. It is quite possible that the syndrome is merely a type of chronic posthemorrhagic anemia complicated by inadequate absorption of iron and perhaps by other dietary deficiencies.

Clinically idiopathic hypochromic anemia resembles pernicious anemia in many ways, although the changes in the blood are entirely different. The two anemias are similar in that both apparently depend on (different) deficiencies of gastric secretion, and both occasionally are familial. In several families idiopathic hypochromic anemia has developed

in females and pernicious anemia in males, and rarely both diseases have developed successively in the same individual. The possibility of a double deficiency must be remembered, although outspoken examples of this are surprisingly rare.

Chlorosis. Chlorosis is described as a disease of unknown etiology, limited to females and occurring chiefly during adolescence, characterized by the development of an anemia of the hypochromic type, and by a prompt response to iron medication. The blood changes are identical with those in idiopathic hypochromic anemia. The chief clinical differences are: (1) the younger age incidence in chlorosis, which usually subsides spontaneously at the age at which idiopathic hypochromic anemia is most frequent; (2) normal or excessive amounts of hydrochloric acid in the gastric juice in chlorosis; and (3) the response of patients with chlorosis to smaller doses of iron. It is probable that chlorosis is due simply to an extreme lack of iron in the diet. Chlorosis has become rare in all countries and has practically disappeared in the United States.

Anemia Due to Deficiency in Copper. In animals (cattle, sheep, rats, swine) a diet grossly deficient in copper as well as in iron produces an anemia which does not respond to the administration of iron alone, but which improves promptly if copper is also given. A minute amount of copper is apparently necessary for the utilization of iron. It is very doubtful, however, whether a significant deficiency in copper occurs naturally in man, even on poor diets, except possibly in young children.

Anemias Related to a Deficiency of the Antianemic Factor in Liver

These anemias are macrocytic, and usually hyperchromic in type. They include: (1) Primary pernicious anemia (by far the most frequent and important). (2) Some cases of sprue, idiopathic steatorrhea, and other chronic intestinal disturbances (fistulae, multiple anastomoses, chronic obstruction). (3) *Diphyllobothrium latum* (fish tapeworm) infection. (4) Cancer of the stomach (rare). (5) Complete resection of the stomach. (6) Chronic diseases of the liver. (7) Pregnancy (rarely). (8) Tropical megalocytic anemia.

Recent work by Minot, Castle, and many others has shown that the normal development and maturation of red cells is dependent on the activity of a specific substance which is commonly called the *antianemic principle of liver,* or erythrocyte-maturing factor. The production of this antianemic principle depends upon the interaction of two other substances: (1) an *extrinsic factor* which is furnished by the diet and is abundant in muscle and in yeast, rice polishings, eggs, milk, and liver (not identical with any of the recognized fractions of the vitamin B_2 complex); and (2) an *intrinsic factor,* which is present in normal gastric juice (possibly secreted also in the proximal part of the duodenum), and presumably is a ferment, although it is not identical with any of the previously recognized gastric ferments. The effective utilization of this antianemic principle depends also (3) upon adequate absorption from the gastrointestinal tract, and probably (4) upon its storage by the liver, and presumably upon its orderly release by the liver and distribution to the hemopoietic tissues as it is needed. A disturbance of any one of these functions, whatever the cause, tends to produce an anemia which is macrocytic and hyperchromic in type. A macrocytic anemia may occur, however, in diseases which are not associated with a disturbance of the anti-pernicious anemia principle (leukemia, primary aplastic anemia, etc.).

In pernicious anemia the disturbance is due to a partial or complete lack of the intrinsic factor in the gastric juice. This is associated with a marked atrophy of the mucosa of the fundus, and a disappearance of the chief and parietal cells, but without notable changes in the pyloric region. In sprue and allied conditions deficient absorption is probably the usual cause. In chronic liver disease, impaired capacity to store or possibly to elaborate the material may be at fault. In some cases, as in certain tropical anemias, there may be a lack of the extrinsic factor in the diet. In pregnancy there is probably an increased demand together with a relatively inadequate formation of the substance. Any of these anemias is usually relieved by supplying adequate quantities of the preformed active material, as by feeding liver or by injecting parenterally suitable extracts of liver. Various British investigators have emphasized the fact that the administration of marmite (an autolyzed extract of brewers' yeast) is as effective as liver in those cases in which the anemia is due to lack of the extrinsic factor, in many cases of sprue and idiopathic steatorrhea, and to a limited extent in some cases of pernicious anemia.

The active principle as it is obtained from liver differs in its thermostability and in other properties from that present within the gastrointestinal tract ("addisin," "hemopoietin"), and in the stomach tissue ("ventriculin"). Where this elaboration occurs is not known. That the liver serves as a storehouse for the substance seems certain, and it probably participates in its production. It has been demonstrated in the liver of patients dying of unrelated diseases by injecting suitable extracts of such livers (autopsy material) into patients with pernicious anemia and observing a reticulocyte crisis, but it is absent from the liver of patients dying of untreated pernicious anemia. It also disappears from the otherwise normal livers of gastrectomized swine, and of swine in which sprue has been experimentally produced.

The chemical nature of the active material has not been determined, although potent highly concentrated preparations have been obtained (e.g., anahemin of Dakin and West). Strandell reported one preparation which was effective in a dose of 2 mg.

Although the changes in the blood are usually more characteristic and more marked in degree in untreated cases of pernicious anemia than in the other conditions mentioned, in some cases of the latter (e.g., sprue, fish-tapeworm infection) they may be indistinguishable. In all these conditions the bone marrow shows megaloblastic hyperplasia in varying degree.

Achrestic Anemia. Israëls and Wilkinson (1936) reported under this term a group of cases in which severe macrocytic anemia, hyperplasia of the bone marrow, but free hydrochloric acid in the gastric juice were manifest. The patients did not respond to liver extract. The erythrocyte maturing factor has been found in the liver in fatal cases. The anemia was attributed to a defect in the marrow which rendered it unable to utilize the active principle. It is doubtful whether this group can be separated from other types of refractory anemia of unknown cause.

Pernicious Anemia. Pernicious anemia is characterized clinically by an insidious onset, usually in adults of middle age; by the gradual development of a severe anemia, with weakness, dyspnea, and other symptoms of hemoglobin deficiency; by a protracted course, marked by remissions and exacerbations; and (in untreated cases) by a fatal termination.

During the active periods of the disease *digestive disturbances* are common: anorexia, gaseous distention, epigastric discomfort, sometimes nausea and vomiting, or diarrhea, and occasionally crises of sharp colicky abdominal pain. Sore mouth and sore tongue due to a stomatitis and *glossitis* are common complaints. There is nearly always *atrophy of the papillae of the tongue*, which presents an abnormally clean, smooth, polished appearance. In the active stages there is fever, associated with evidences of increased blood destruction.

Paresthesias of the extremities nearly always develop, and are often an early symptom. Focal degenerations of the spinal cord (*combined sclerosis*) are common. They occur (1) in the posterior columns, causing *ataxia,* weakness, and minor sensory disturbances, particularly *loss of the vibratory sense* over the lower legs, diminished reflexes, occasionally hyperesthesias; (2) in the lateral columns, causing *spasticity,* exaggerated reflexes, and less often sphincter disturbances. These changes are not proportional to the anemia, and may antedate it. Rarely combined sclerosis occurs in patients who do not develop an anemia, although as in typical pernicious anemia they show an achlorhydria, and usually some degree of macrocytosis. Combined sclerosis is extremely rare in the other related anemias. Peripheral neuritis also occurs and may account for the paresthesias in some cases. Minor cerebral disturbances are common.

In practically all cases there is a *gastric achlorhydria,* even after histamin injection. The volume of gastric secretion is scanty and is increased but little by histamin. Mucus is scanty. The ordinary ferments are usually diminished, and often absent (true achylia). The gastric deficiency is permanent, even in well-treated patients. A few rare cases have been reported in which free hydrochloric acid was present in the gastric juice of the patient. The intrinsic factor has been absent in patients of this group who have been tested as to this point. There is some evidence to indicate that the lack of intrinsic factor in pernicious anemia, in some cases at least, is relative rather than absolute. It has been suggested, without as yet definite proof, that variations in the amount of intrinsic factor secreted may account for the fluctuations in the course of the disease.

The *blood* shows a marked reduction in the red-cell count, frequently to 2.0, rarely to 0.5 million or less. The hemoglobin is relatively less reduced, so that the color index and mean hemoglobin content of the cells are increased. The volume index and mean corpuscular volume are also increased, more regularly and often more markedly than is the color index. The hemoglobin concentration in the cells is normal or slightly reduced. Anisocytosis is marked, and in severe cases it becomes more pronounced than in any other anemia. Poikilocytes, microcytes, and macrocytes are numerous. Large, oval, deeply staining cells are highly characteristic, and a few are usually present in the early stages of the disease and during the remissions. The mean diameter is increased (to 8.5 to 9μ), and the cells are dark (the thickness is also increased).

A few *normoblasts* are present in most of the patients with marked anemia. Typical *megaloblasts* are present at some stage of the disease in untreated patients, but they may be hard to find. They can rarely be found after treatment with liver, even though this is inadequate in quantity. During the *blood crises* which occasionally occur in untreated patients, there is a sudden outpouring of normoblasts and megaloblasts, reticulocytes, polychromatophilic cells, and cells with nuclear particles.

The platelets are reduced. There is a leukopenia. A few myelocytes are often present, but on the other hand there is a "shift to the right" in the sense of Arneth, with occasional huge neutrophils containing hypersegmented nuclei with 6 to 10 lobes, "pernicious anemia neutrophils."

During the exacerbations of the disease there are mild jaundice, an increase in

the bilirubin in the serum, and all the other characteristic features of a hemolytic anemia. It is generally believed, however, that this *increased blood destruction* is not a primary cause of the anemia, but is the result of the entrance into the circulation of imperfectly formed cells which fall ready victims to the normal physiologic processes for the removal of defective cells.

According to Minot, whose views are widely accepted, the fundamental disturbance in pernicious anemia is inadequate cell formation. Although the marrow is hyperplastic, the megaloblasts are unable to complete their development and produce erythrocytes. There is an arrest of maturation, which is relieved by the administration of the active principle of liver.

This view has been questioned, however, notably by Dobriner and Rhoads (1938), who attempted to measure the rate of cell formation and destruction in various stages of the disease. They utilized the rate of excretion of urobilin as a measure of red-cell destruction, and that of coproporphyrin I as a measure of red-cell formation. Although this substance is not directly concerned in the production of hemoglobin, they believe it is a constant by-product of the synthesis of coproporphyrin III, which is a constituent of hemoglobin, and that it can be used to measure the rate of formation of the latter. In pernicious anemia during a relapse they found cell (hemoglobin) production actually accelerated, as well as cell destruction. During a remission the rate of cell destruction fell to normal, and the rate of cell formation was also somewhat reduced. They believe, therefore, that lack of erythrocyte-maturing factor results in increased hemolysis rather than in a quantitative reduction in cell output.

In patients with a red-cell count below 3.5 million adequate liver therapy is followed by a transient *reticulocyte crisis,* which begins on about the third day, and reaches a peak on about the seventh or eighth day. The height of the peak varies inversely with that of the initial red-cell count.

With a red-cell count of 1.0 million the reticulocytes should reach 35 to 40 per cent; with 2.0, about 20 per cent; and with 3.0, about 5 per cent. A lesser rise indicates either a mistaken diagnosis, inadequate dosage, or some complicating disease. If the initial dose of active principle has been too small, as indicated by an inadequate reticulocyte response, an increase in the dose will be followed by a second reticulocyte crisis. However, if the initial dose was adequate, a further increase will have no significant effect on the reticulocytes. These observations have proved to be of great practical value in the control of treatment. In favorable cases the red-cell count may rise 2.0 million in one month, normal figures will be attained after two to three months, and the qualitative abnormalities largely disappear except for the persistence of a few macrocytes. The reappearance of these abnormalities or a fall in the red-cell count indicates that the maintenance dose of liver is inadequate.

The rise in the red-cell count during the first two weeks is fully as significant as the rise in reticulocytes and in some cases is adequate in spite of a subnormal reticulocyte response. Della Vida has devised a formula to gauge the adequacy of this rise.

$$I = 0.93 - 0.214\ Eo$$

I indicates the average weekly rise in the red-cell count in millions during the first two weeks of treatment, and Eo is the initial red-cell count in millions.

Failure to secure a satisfactory response is often due to deficient absorption from the gastrointestinal tract. In such cases excellent results can be obtained by *intramuscular injections*, which on the average are at least 50 times as effective as the administration of

equivalent doses by mouth. With the best preparations a maximum response follows the daily intramuscular injection of extract from 15 to 20 Gm. of liver. A normal count can usually be maintained by a similar dose given once a week, and in some cases once a month. Patients with combined sclerosis require far larger doses, continued for many months. To control this process it may be necessary to give two or three times the amount which suffices to restore and maintain a normal red-cell count. Improvement at best is slow, and is limited by the degree to which irremediable atrophy of nerve cells has occurred.

Dried defatted stomach administered by mouth is as effective as liver so administered.

Folic Acid. Folic acid (*L. casei* factor), either derived from natural sources (liver, yeast, etc.) or synthesized (pteroylglutamic acid), causes a reticulocyte response and a rise in the red cell count in pernicious anemia and the related macrocytic anemias which appear to be essentially identical with those produced by potent liver extract. Folic acid is active either on oral or parenteral administration, and it is usually effective in a dose of 5 to 10 mg. per day. It is not yet known whether it is as satisfactory as liver extract in maintaining remissions and particularly in preventing degenerative changes in the nervous system.

The mechanism of its action and its relation to the antianemic principle of liver are not known. It cannot be identical either with the latter or with the intrinsic factor. It is apparently not identical with the extrinsic factor as the latter is usually defined, although there may be a relationship between them.

In several species of animals a diet deficient in folic acid has resulted in the appearance of a severe macrocytic anemia, often associated with a leukopenia and thrombocytopenia.

Although much has been learned regarding the pathogenesis of pernicious anemia, the underlying cause of the defective gastric secretion remains obscure. In some cases it is a familial constitutional defect. Many families have been reported in which two or more members have had pernicious anemia, or in which other members have had achylia. Most patients with pernicious anemia are sthenic in type and have a light complexion, with fine, often prematurely gray hair. However, the disease may (rarely) occur in negroes.

The theory that a chronic *dietary deficiency* plays a part is attractive and receives some support from the experiments of Miller and Rhoads (1935). By feeding swine a suitably deficient diet, they produced a diseased state closely resembling tropical sprue and (less closely) pernicious anemia in man. These animals showed an anemia (usually macrocytic), with gastrointestinal disturbances and stomatitis, the intrinsic factor disappeared from the gastric juice and the antianemic principle from the liver, and the bone marrow showed megaloblastic hyperplasia. The condition responded to injections of liver extract.

In members of the poorer classes in Africa and the Orient, however, who subsist on grossly inadequate, unbalanced diets and who often show marked nutritional anemias which may be macrocytic, typical pernicious anemia is rare. If a dietary deficiency be the cause of pernicious anemia, either it must be a highly specific one, or more probably it must operate in conjunction with other factors not yet recognized.

Tropical Megalocytic Anemia. This is a disease described as occurring in the native population of west Africa, India, and China (Wills and Mehta, 1930). It occurs chiefly in women between 20 and 30, and is often precipitated by pregnancy. The symptoms are those of any severe anemia. Edema is often marked. There may be a glossitis, but marked gastrointestinal disturbances are exceptional, and achlorhydria is rare. The blood shows a macrocytic hyperchromic anemia which may be severe, with marked anisocytosis and many megalocytes, but without an increase in serum bilirubin, and but few poikilocytes and polychromatophilic cells. It is believed to be due purely to dietary deficiency (in extrinsic factor), as it is cured permanently by marmite. It is cured by oral or parenteral administration of the cruder types of liver extract, but not by highly concentrated extracts (Wills and Evans, 1938). Recent studies suggest that a deficiency of folic acid plays a major part in the production of such anemias.

Sprue. Sprue is a tropical or subtropical disease of unknown origin occurring most frequently in India, China, and the East Indies, where it is apt to attack white immigrants from temperate latitudes. It also occurs in the West Indies and has been observed in the southern United States. It occurs chiefly in adults, more frequently in women. It is characterized by the gradual development of a chronic morning diarrhea with progressive emaciation, weakness, and anemia. The *stools* are typically bulky, grayish, pultaceous, and frothy, and contain large amounts of fat, chiefly as fatty acids. Glossitis and marked atrophy of the mucous membrane of the tongue and the entire gastrointestinal tract occur, associated with great flatulent distention. Free hydrochloric acid is present in the gastric juice in many cases, and (if absent) may return during a remission. The intrinsic factor of Castle has been present in some cases and absent in others. There are no bone changes and no gross disturbance of calcium metabolism.

Anemia is present in most cases, but varies much in severity (red-cell count usually about 3.0 million, but may fall below 1.0 million). It is usually macrocytic and mildly hyperchromic in type, resembling pernicious anemia except that the abnormalities are less marked. The anemia often responds to yeast, and regularly to liver extract, which in large doses also relieves the buccal and gastrointestinal disturbances. Spies and others have reported marked improvement after administration of folic acid. The anemia is probably due mainly to defective absorption of the antianemic principle, or in some cases, to lack of the intrinsic factor. In a minority of the cases it is hypochromic, and responds to iron but not to liver.

Idiopathic Steatorrhea (Celiac Disease, Nontropical Sprue). This is a disease of temperate climates which usually begins in infancy or childhood, although it may not be recognized until adult life. It is characterized (1) by chronic diarrhea, with abdominal distention, and fatty but not frothy stools; (2) by a disturbance of metabolism associated with defective absorption of calcium salts and vitamin D, and characterized by osteoporosis, pains in the bones, bone deformities, and pathologic fractures; and (3) by emaciation and anemia, which are often severe. Achlorhydria is rare. Glossitis may occur, and some degree of atrophy of the lingual papillae is not uncommon. Fecal excretion of calcium is increased, the blood calcium is often low, and latent tetany is common. Lenticular opacities may occur, and occasionally cutaneous eruptions suggestive of pellagra.

The anemia is usually hypochromic in type, particularly in children. The color index is low, and anisocytosis is marked. The average cell diameter is usually within normal limits, but in some cases it is distinctly increased. Rarely numerous normoblasts have been present. The anemia (but not the other symptoms) is relieved by iron, and it is attributed to defective absorption of iron.

In other cases, particularly in adults, the anemia is macrocytic and mildly hyperchromic, resembling that commonly seen in sprue. In this type the anemia responds to the administration of liver, or large doses of yeast preparations, and is attributed to defective absorption of the antianemic principle. The gastrointestinal disturbances are also usually relieved by large doses of liver extract, and many believe the condition is identical with tropical sprue.

Anemia Due to Diphyllobothrium Latum Infection. *Diphyllobothrium latum* causes anemia in only a very small proportion (about 1 : 1000) of the infected individuals. In these cases it may be severe and practically indistinguishable from pernicious anemia, except that combined sclerosis rarely if ever occurs. The anemia is sometimes cured permanently simply by expulsion of the worm. It is also cured by liver therapy, but this must be con-

tinued until the worm is expelled. Free hydrochloric acid is usually absent but may return after expulsion of the worm. Hernberg (1936) reported finding the intrinsic factor.

Anemia Due to Cancer of the Stomach. Cancer of the stomach often causes anemia, which is almost invariably hypochromic and microcytic in type. In the absence of bleeding it is usually moderate in degree, but may be extreme (R.B.C. 1.0, Hb. 15 per cent), and quite like that in idiopathic hypochromic anemia. In rare instances a macrocytic hyperchromic anemia develops which may be indistinguishable from pernicious anemia. This may temporarily improve under liver therapy, and is attributed to loss of the intrinsic factor. The anemia which follows extensive operations on the stomach also is more often hypochromic than hyperchromic in type.

On the other hand, the development of carcinoma of the stomach during the course of pernicious anemia is relatively common. Kaplan and Rigler (1945) in autopsy studies found carcinoma of the stomach three times as frequent in cases of pernicious anemia as in others in the same age group. The pathologic changes in the stomach which result in pernicious anemia appear to favor the development of carcinoma.

Anemia Due to Myxedema. Myxedema frequently causes an anemia which may be either hypochromic or hyperchromic in type. The latter type of anemia is relieved by liver therapy, and is attributed to a lack of intrinsic factor, which may be temporary (relieved by administration of thyroid alone), or permanent (requiring continuous administration of liver, which controls the anemia, but not the myxedema).

Anemia Due to Pregnancy. Pregnancy is so frequently associated with a mild hypochromic type of anemia that some have regarded this as physiologic. This usually increases gradually from the third to the seventh month, after which there may be some spontaneous improvement. The red-cell count not infrequently falls to 3.5 million, and the hemoglobin to 50 to 60 per cent. Occasionally much lower figures are observed. The anemia responds well to iron, and is due to an iron deficiency. The latter is probably due in part, at least, to poor absorption, associated with the hypochlorhydria or achlorhydria which is commonly observed during pregnancy. It is partly the result of an increased need for iron to supply the fetal tissues. The apparent degree of the anemia is somewhat exaggerated by the hydremia which is present. The anemia usually subsides after delivery. Failure to do so suggests the presence of idiopathic hypochromic anemia, which is markedly aggravated by pregnancy. There is apt to be a recurrence in subsequent pregnancies.

In relatively rare instances a hyperchromic macrocytic anemia develops. The changes in the blood resemble closely those in pernicious anemia. There are evidences of increased blood destruction. The disease is severe, runs a relatively acute course, without remissions, and is often fatal if untreated. It may appear during the puerperium, but spontaneous recovery may occur after delivery. It responds well to liver (frequently also to transfusions), and recovery is usually permanent. It may not recur during subsequent pregnancies. There is usually free hydrochloric acid in the gastric juice, but subacidity is common. Lack of the intrinsic factor—presumably temporary—has been reported in a number of cases. Strauss reported two patients who developed pernicious anemia later.

Anemias Related to a Functional Insufficiency of the Bone Marrow (The Aplastic or Aregeneratory Anemias)

The inadequacy is usually relative, but in rare instances there appears to be a virtual cessation of red-cell formation. The marrow as a rule is aplastic to a greater or lesser degree. In some cases, however, it shows marked hyperplasia, and there appears to be an arrest in the maturation of the cells. Among the more important conditions in which anemia of this type occasionally occurs are the following:

1. In newborn infants as a rare constitutional defect.
2. As a terminal phenomenon in anemias of other types, as in chronic posthemorrhagic anemia, pernicious anemia, and the myelophthisic anemias.
3. Anemias secondary to infection, particularly to such chronic infections as subacute bacterial endocarditis, chronic infectious arthritis, chronic pyogenic infections, and oral sepsis. These are often simple microcytic in type.
4. In advanced chronic nephritis (usually normochromic in type).
5. Malignant disease, in most of those cases in which hemorrhage does not play a part.
6. Dietary insufficiencies, other than simple lack of iron, including the avitaminoses, such as scurvy, etc.
7. Chronic poisoning with such chemicals as benzol, trinitrotoluol, arsenic, gold or radium salts (Martland, 1931).
8. After excessive exposure to roentgen-rays or radium.
9. Acute idiopathic aplastic anemia.
10. Primary splenic panhematopenia.
11. The myelophthisic anemias.

This list manifestly includes many heterogeneous unrelated conditions, and the changes in the blood which they show vary in detail. A high grade of anemia may occur in all of them. In the milder cases some new-cell formation persists, and the blood shows imperfectly formed cells and immature red cells in small numbers. The blood often resembles that in cases of posthemorrhagic anemia, except that the hypochromia and microcytosis are usually less marked. In severe advanced cases the appearance of the blood is more distinctive. Despite a marked degree of anemia, the individual red cells which remain are relatively normal. Anisocytosis is slight, and the color index and volume index are usually within normal limits. The anemia is "normocytic" and "normochromic." Reticulocytes and other immature red cells are entirely absent. There is a neutrophilic leukopenia and a reduction in platelets which may be extreme, and associated with a (symptomatic) purpura hemorrhagica. There is no evidence of increased blood destruction.

Idiopathic Aplastic Anemia. In idiopathic aplastic anemia these features are seen in maximum degree. This is a rare disease of unknown cause, occurring chiefly in adolescents and young adults, characterized by progressive weakness and prostration, fever, rapidly developing anemia, extreme leukopenia, thrombocytopenia, purpura, and bleeding, and a fatal outcome within a few weeks or months. It is the "aleukia" of German writers. The spleen is not enlarged. The gastric juice is normal. The bone marrow in typical

cases is markedly aplastic. Cases which are clinically indistinguishable, however, have shown marrow which is normally cellular or even hyperplastic, and in some of them reticulocytes, normoblasts, myelocytes, and myeloblasts have appeared in the circulating blood. Krumbhaar has suggested the term "pseudoaplastic anemia" or "progressive hypocythemia" for such cases.

Idiopathic aplastic anemia has been confused with pernicious anemia, and (more reasonably) with idiopathic purpura and acute leukopenic leukemia. Cases of severe poisoning with benzol, radium, etc., may present an identical picture.

Scurvy. Scurvy often causes an anemia which is usually moderate in degree but may be severe (R.B.C. 2.0 million, Hb. 30 per cent). It is hypochromic and usually microcytic in type. It cannot be explained by the (relatively trivial) hemorrhages which occur. It is not influenced by iron or liver but responds to the ingestion of an adequate diet. The view that the anemia is a specific manifestation of a deficiency of vitamin C has been seriously questioned.

Myelophthisic Anemia. This is a term applied to those conditions in which the erythropoietic tissue of the marrow is invaded and more or less crowded out by other tissue. Except in extensive leukemic infiltration, the bulk of the invading tissue is rarely sufficient quantitatively to explain the anemia on the basis of simple mechanical displacement, and a functional inadequacy of the remaining marrow must be assumed. These include:

1. Osteosclerotic anemia.
2. The leukemias.
3. Some cases of Gaucher's disease, and allied conditions.
4. Metastatic tumors in the bone marrow (frequently).
5. Multiple myelomas (occasionally).
6. Hodgkin's disease (rarely).

In many cases the anemia is slight or moderate in degree, but it may be severe. Anisocytosis is often marked, but the color index and volume index are usually about normal or moderately reduced. In many (but not all) cases the blood shows changes suggesting intense stimulation (or irritation) of the marrow. There are numerous immature erythrocytes, including many normoblasts and macroblasts, and even megaloblasts. There is a neutrophilic leukocytosis, with myelocytes in varying number, and occasionally a few myeloblasts. They are sometimes classed as *erythroblastic anemias* (Vaughan). Areas of hyperplastic erythropoietic tissue are commonly found in the long bones, or sometimes in the spleen and other extramedullary tissues.

Osteosclerotic Anemia. This is a term usually applied to the condition described by Albers-Schönberg as "marble bone" disease. This is a rare disease of unknown cause, which may be dependent on a constitutional abnormality of bone development, and is sometimes familial. It begins in early life, although it may not be recognized until the third decade. It is characterized by a peculiar type of irregular thickening of the bones, involving especially the shafts of the long bones. The new bone encroaches on and eventually may largely obliterate the marrow, which shows in addition extensive fibrosis. In spite of the thickening, the bones are fragile, and gross deformities and pathologic fractures are common. In the later stages a severe anemia develops (as low as R.B.C. 0.82 million, Hb. 10 per cent, Vaughan), characterized by a marked erythroblastosis. The spleen and liver are much enlarged as a result of myeloid hyperplasia.

Myelosclerosis. This is a term applied by Mozer (1927) to a condition developing in adults somewhat similar to osteosclerotic anemia, in which the bones become abnormally dense but not widened, and the cellular marrow is replaced by fibrous tissue. Eventually a severe anemia develops, with erythroblastosis and splenomegaly.

ANEMIAS ASSOCIATED WITH ACCELERATED BLOOD DESTRUCTION (HEMOLYTIC ANEMIAS)

Although accelerated blood destruction is a prominent feature of these diseases, in many of them inadequate blood formation is also important in the production of the anemia. In some of them, as in pernicious anemia, sickle cell anemia, and probably hemolytic jaundice, the primary disturbance appears to be the formation of red cells which are inherently defective, and thus susceptible to the normal processes for removal of damaged cells. There is no conclusive evidence that anemia is produced by removal and destruction of normal cells, because of a pernicious overactivity of the reticulo-endothelial tissues.

In nearly all cases red cells which are defective or which have been damaged are removed from the circulation before hemolysis occurs. The mechanism of this process has been described in the previous chapter. If the rate of red-cell destruction is accelerated (or if the liver is injured), the liver may fail to remove the bilirubin from the serum as fast as it is formed. As a result bilirubin accumulates in the plasma and gives the latter a yellow color. The icterus index rises, and the serum gives a positive (indirect) van den Bergh reaction. The skin and sclerae become more or less jaundiced. In some cases this may be deep, but usually it is relatively slight, and the color is a pale lemon-yellow rather than the orange tint of obstructive jaundice. The difference, however, is purely a quantitative one. The color in both cases is due to bilirubin. The urine becomes dark-colored, and contains increased amounts of urobilin and urobilinogen, but no bilirubin (or only traces). The sediment may show renal epithelial cells containing hemosiderin granules. The urobilin in the feces is increased. At autopsy the amount of iron in the liver and spleen is increased. These phenomena are marked only during periods of rapid blood destruction. If the latter is relatively slow, they will be slight, and perhaps limited to a small increase in the bilirubin in the serum.

The rapid destruction of red blood cells in the body, regardless of the disease in which it occurs, gives rise to a characteristic clinical syndrome. There is fever, sometimes a chill, weakness and prostration, pain in the back, and crises of acute colicky abdominal pain, with nausea, vomiting, and jaundice. The symptoms may simulate those of various acute abdominal conditions, and have led to unnecessary operations.

In rare instances in which a large number of red cells are rapidly destroyed, the cells may be hemolyzed in the circulation and the hemoglobin liberated into the plasma (*hemoglobinemia*). If the amount of hemoglobin so liberated is large (when about one-sixtieth or more of the red cells are abruptly destroyed and the concentration of hemoglobin in the plasma reaches about 135 mg. %), *hemoglobinuria* occurs.

Fairley has shown in blackwater fever and other diseases in which there is hemoglobinemia that much of the extracorpuscular hemoglobin is converted in the plasma into *methemalbumin*, a combination of hematin with plasma albumin. This is not excreted in the urine but is removed by the liver. It can be identified spectroscopically, preferably by Schumm's method. A little ether is layered over a measured volume of serum and one-

tenth this volume of concentrated ammonium sulfide together with a few drops of ammonia are pipetted into the serum. The tube is shaken and examined directly for an absorption band in the yellow-green (at 568 mμ; see p. 813, hemochromogen).

Hemoglobinuria is met with: (1) after transfusions of incompatible blood, (2) in paroxysmal hemoglobinuria, (3) in blackwater fever, (4) rarely in severe infections and intoxications of the types enumerated below (as in gas bacillus gangrene, and poisoning with arseniuretted hydrogen), and in favism, (5) in march hemoglobinuria, and (6) in paroxysmal nocturnal hemoglobinuria.

A *hemolytic anemia,* usually without notable hemoglobinemia or hemoglobinuria, is met with chiefly in the following conditions:

1. Some acute infections, as gas-bacillus gangrene, sepsis, especially puerperal sepsis and other streptococcal infections, typhoid fever, malaria, and Oroya fever. Rarely a profound anemia may develop within a few hours.
2. Some cases of acute poisoning with certain drugs: phenol, benzol, and their derivatives, nitrobenzene, trinitrotoluene, phenylhydrazine, aniline, acetanilid, sulfonamides; saponin; potassium chlorate; lead and other heavy metals; arseniuretted hydrogen; snake venom; etc.
3. Rarely in leukemia, Hodgkin's disease, carcinomatosis.
4. Extensive burns.
5. Idiopathic cases, including the acute febrile hemolytic anemia of Lederer.
6. Favism.
7. Pernicious anemia and related macrocytic anemias during acute exacerbations of the disease.
8. Familial hemolytic jaundice.
9. Sickle cell anemia.
10. Icterus gravis neonatorum.
11. Cooley's erythroblastic (Mediterranean) anemia.

The morphologic changes in the red cells in anemias of this type are not distinctive, except in the specific diseases pernicious anemia, hemolytic jaundice, and sickle cell anemia. In severe cases spherocytes may be found. Anisocytosis and anisochromia are usually moderate and are typically less than in chronic posthemorrhagic anemia of the same degree. The volume index (mean corpuscular volume) and color index may be somewhat reduced, but more often they are within normal limits, and occasionally they are increased. These anemias are not hypochromic, probably because the iron from the cells which have been destroyed is retained in the body and is readily available for the production of new hemoglobin. Evidences of active red-cell formation, reticulocytes, polychromatophilic or stippled red cells, even normoblasts, are usually present and may be numerous, as in lead poisoning. In acute cases there is usually a leukocytosis and an increase in platelets. In protracted chronic cases the blood may finally assume the features of an aplastic type of anemia.

Paroxysmal Hemoglobinuria. This is characterized clinically by recurring brief paroxysms of chills, fever, pain in the back, prostration, cramps, vomiting or diarrhea, numbness in the extremities, and hemoglobinuria. The individual attacks are usually precipitated by exposure to cold, or experimentally by holding the arm in ice water. They are followed by jaundice and (hemolytic) anemia which may be severe (R.B.C. 1.0 million).

The hemolysis is brought about by an hemolysin of the usual complex type. The stable constituent ("amboceptor") is peculiar in that it will combine with cells only at a low

temperature. Once combined, it renders the cells susceptible to the lytic action of complement when warmed to body temperature (the *Donath-Landsteiner phenomenon*).

No.	*Serum, 0.5 ml.*	*Cell suspension, 0.2 ml.*	*Complement, ml.*	*Salt Sol., ml.*	*Positive Result*
1	Patient's	Patient's	0.2	0.1	Hemolysis
2	Control	Control	0.2	0.1	0
3	Patient's	Control	0.2	0.1	Hemolysis
4	Control	Patient's	0.2	0.1	0
5	..	Patient's	0.2	0.6	0
6	..	Control	0.2	0.6	0

The *hemolysin* can usually be demonstrated in the following simple manner. About 1 ml. of fresh (warm) blood is placed in each of two test tubes and kept at body temperature until the serum has separated. One tube is left in the water bath at 37° C. as a control and the other is put in ice water for five to seven minutes. Then this is placed in the water bath for an hour. If the serum in the control tube remains colorless, the presence of the hemolysin is indicated by more or less tingeing of the serum in the second tube. If the result is doubtful, a little additional complement is added to each tube and the tubes returned to the water bath. (A 1 : 10 dilution of fresh guinea-pig serum, or fresh normal human serum of the same blood group is used; this is usually necessary if the blood has been chilled for more than 10 minutes.)

For more precise demonstration about 10 ml. of the patient's blood is withdrawn in a warm syringe, about 8 ml. of the blood is allowed to clot (for serum) and the rest is oxalated. The blood, solutions and apparatus must be kept at body temperature throughout all manipulations. The cells are washed and a 5 per cent suspension is prepared. Ten ml. of normal human blood of the same blood group is secured and treated in the same way. A 1 : 10 dilution of fresh guinea-pig serum is prepared as complement. Tubes are set up as shown above.

All tubes are placed in ice water for 10 minutes and then in the water bath at 37° C. for half an hour. A positive reaction is indicated by hemolysis in tubes (1) and (3) and by absence of hemolysis in all the other tubes.

An attack may be precipitated by immersing the hands or feet in ice water for 10 to 20 minutes or by putting a ligature around a finger and holding this in ice water.

The condition is regarded as a rare late manifestation of *syphilis,* since a large proportion of patients give a positive serologic reaction, and it can sometimes be cured by adequate treatment of the syphilis. The hemolysin has been observed in some patients with late syphilis, who show no clinical symptoms of paroxysmal hemoglobinuria, but not in other conditions. The disease must be differentiated from blackwater fever and other rare forms of hemoglobinuria.

March hemoglobinuria may follow unusual muscular exertion, such as long marches or standing for hours in a lordotic posture. The quantity of red cells actually destroyed is relatively small, and significant anemia rarely if ever occurs.

Paroxysmal Paralytic "Hemoglobinuria." This disease, similar to a relatively common disease of horses, has been reported in a few human beings. There are recurring attacks of extreme muscular weakness, followed by hemoglobinuria, and later by more or less marked muscular atrophy but little or no anemia. The pigment excreted is *myoglobin.* The muscles in fatal cases show marked degenerative changes and loss of pigment ("fish flesh"). A somewhat similar acute degeneration of striated muscle with myoglobinuria has been observed in Königsberg ("Haffkrankheit") in individuals (and in cats) who had eaten fish which had ingested poisonous resinous acids, the waste products of cellulose factories. Myoglobinuria has also been observed in cases of *crush-syndrome.*

Table 49

SPLENOMEGALY IN DISEASES OF THE BLOOD: DIFFERENTIAL DIAGNOSIS

	Time of Onset	*Duration*	*Splenomegaly*	*Pathology of Spleen*	*Splenectomy Beneficial*	*Hemorrhages*	*Anemia*	*Leukocyte Count*
Pernicious anemia	Adult late	Many years	Slight, occasional	Fibrosis	No	Rare	Severe	Low
Idiopathic hypochromic anemia	20–50	Many years	Slight in 25–30%	?	No	Rare	Moderate to severe	Normal or low
Familial hemolytic jaundice	Congenital or childhood	Life	++	Congestion pigmentation	Usually curative	Rare	Slight to severe	Variable
Acquired hemolytic jaundice	Adult	Many years	++	Congestion pigmentation	Often	Rare	Severe	Variable
Banti's disease	Childhood; young adult	Several years	+	Hyperplasia fibrosis	Often	Gastric common	Slight to severe	Low
von Jaksch anemia	Infancy	Several months	++	Hyperplasia fibrosis	In some cases	Rare	Often severe	Increased
Idiopathic thrombopenic purpura	Variable often in childhood	Many years	Rare	None characteristic	Often curative	+++	Severe after bleeding	Variable, often reduced
Anaphylactoid purpura	Childhood	Brief attacks	+ in some cases	None characteristic	No	Trivial	No	Increased
Polycythemia	Adult late	Several years	+	Congestion hyperplasia	Probably harmful	Occasional	No	Increased
Chronic myeloid leukemia	Adult	2–5 years	+++	Myelocytic infiltration	Harmful	Occasional	Becomes severe	Very high
Chronic lymphatic leukemia	Adult	2–10 years	++	Lymphocytic infiltration	No	Rare	Becomes severe	Usually very high
Acute leukemia (myelogenous, lymphatic, monocytic)	Childhood; youth	A few months	+	Primitive cell infiltration	Harmful	Common ++	Severe	Low, normal or high
Hodgkin's disease	Any age	1–5 years	++	Peculiar hyperplasia, fibrosis	No	No	Becomes severe	Variable
Lymphosarcoma	Any age	1–3 years	+ in some cases	Hyperplasia of lymphocytes	No	No	Slight to moderate	Usually normal
Gaucher's disease	Infancy	Life	+++	Hyperplasia of R.E. (foam) cells	No	Occasional	Slight	Normal or low

Paroxysmal Nocturnal Hemoglobinuria. This is a chronic relapsing disease of unknown origin, characterized by an insidious onset with weakness, anemia, jaundice, and, later, recurring attacks of hemoglobinuria without obvious exciting cause, which usually occur at night and lead to severe anemia. The blood shows a continuous hemoglobinemia, a leukopenia, many reticulocytes, and normal fragility. Ham (1939) reported that the increased hemolysis occurred during sleep and was associated with a fall in the pH of the plasma. The abnormality appeared to be in the cells, since they were hemolyzed in acidified fresh serum, either of the patient or of normal individuals of the same blood group. The sediment of 1 ml. of 5 per cent suspension of washed red cells of the patient is suspended in 1 ml. fresh serum to which has been added 0.05 ml. of N/3 hydrochloric acid; the suspension is incubated at 37° C. Splenectomy has not been beneficial. (Reviewed by Hamburger and Bernstein, 1936.)

In none of these conditions is there any relation to syphilis or to exposure to cold, and the Donath-Landsteiner phenomenon is absent.

Lederer's Acute Hemolytic Anemia. This is a syndrome observed chiefly in children and young adults, and characterized clinically by an acute onset with fever, by the rapid development of a severe hemolytic anemia (R.B.C. to 1.0) with great prostration, jaundice, abdominal pain, vomiting and diarrhea, often purpura and hemorrhages, occasionally anuria and uremia. There is usually a leukocytosis, and often many myelocytes and some myeloblasts appear in the blood. The disease is often fatal if not properly treated, but can usually be cured by prompt and repeated transfusions, or, if these fail, by splenectomy. It cannot be distinguished sharply from other clinical types of hemolytic anemia of unknown cause, and is probably not a disease entity.

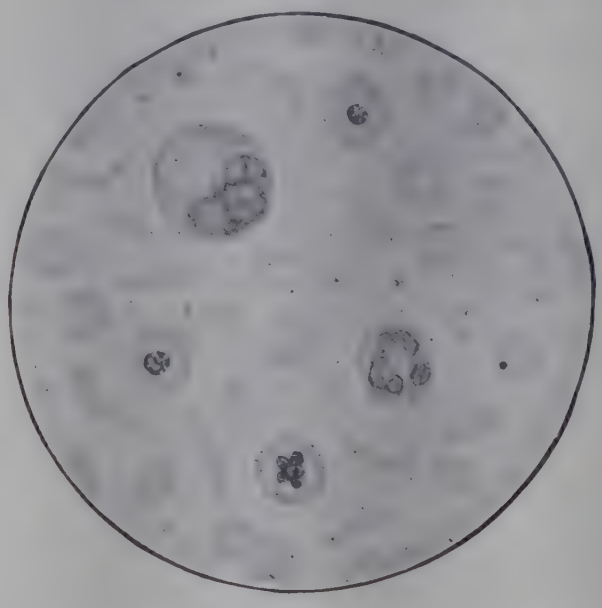
Lead Poisoning. Lead poisoning frequently causes an anemia which is usually hypochromic and moderate in degree (R.B.C. 3.0 to 4.0 million), but may become severe. In severe cases it may cause an acute hemolytic anemia. The most characteristic feature of the blood is the appearance of many reticulocytes and stippled cells. These cells may appear within a few days after exposure to lead, in the absence of appreciable anemia. Although stippled cells may appear in any anemia in which active red-cell regeneration is taking place, and although they may be sparse in some cases of lead poisoning, their early appearance and their presence in large numbers is highly characteristic.

Actual counts of *stippled cells* have been utilized to detect and measure the degree of absorption of lead in industrial workers. In Germany a count of from 100 to 300 per million red cells has been regarded as an indication for enforced change of occupation. Belknap (1935) has found that men with counts of 500 to 1000 per million might continue at work for years without clinical symptoms of lead poisoning, but that an abrupt increase above these figures was usually followed by acute symptoms. Counts of 40,000 per million and more have been observed. The number may be estimated by counting the number of stippled cells in 50 oil-immersion fields in an ordinary thin film, and multiplying this figure by 100 (the average number of red cells per field is about 200, and this should be roughly checked by those not experienced in such counts). McCord's basophilic aggregation test (p. 392) should be used if the cells are sparse.

An increase in *reticulocytes,* although less specific, is an earlier and more sensitive sign of lead absorption than the appearance of stippled cells. There is a rough parallelism between the counts of the two types of cells. Jones (1935) found the number of reticulocytes trebled with 100 stippled cells per million, and five times the normal with 1000 stippled cells per million. Reticulocyte counts of 16 per cent and more have been observed in acute poisoning. Jones found appreciable numbers of normoblasts in about 5 per cent of the chronic cases.

The resistance of the red cells to hypotonic salt solution is increased, but the cells appear to be abnormally fragile and susceptible to mechanical injury (Aub).

Acetanilid. Acetanilid and related drugs in overdose cause a transformation of hemoglobin to *methemoglobin*. This imparts to the mucous membranes a characteristic, dusky, cyanotic tinge. It can be recognized by spectroscopic examination, but must be differentiated carefully from sulfhemoglobin which gives a closely similar spectrum. As a rule, after removal of the poison there is a reversion to normal hemoglobin without much injury to the red cells. More rarely it causes a severe acute hemolytic anemia, associated with a leukocytosis and occasionally an erythroblastosis.



Sickle cell anemia. Severe case showing an unusual number of sickled cells in a stained film. Three normoblasts. (*Upper left*) Macrophage containing a red corpuscle.

Sickle Cell Anemia. This is a hereditary constitutional anomaly practically limited to Negroes, transmitted by either sex as a dominant Mendelian character and distinguished by the tendency of the red cells (in sealed fresh preparations) to assume characteristic bizarre shapes. The cytoplasm of the cells at two or more points becomes drawn out into elongated spinelike projections, so that the cells become crescentic, or more often oat-shaped or irregularly stellate. Hahn and Gillespie found that the cells would resume the normal shape if oxygen was supplied to the preparation, and would again "sickle" if it was withdrawn. These distortions are not seen in ordinary fixed films, except to a slight extent in a few cells in the severe cases. Sickling within the vessels has not been directly observed.

A majority of the individuals who show this trait are symptomless. In a few cases (about 2.5 per cent in the United States) recurring attacks of acute hemolytic anemia occur, with partial recovery in the intervals. In addition to the usual symptoms due to acute hemolysis, older patients often complain of deep-seated pain in the bones and joints, often associated with osteoporosis in roentgenograms; and chronic punched-out ulcers over the lower legs. The skull may show changes similar to those in Mediterranean anemia. Many cases show cardiac dilatation and clinical manifestations of myocardial insufficiency. Peripheral thromboses are frequent. The spleen is often enlarged in young children; later it becomes small and fibrotic. Symptoms appear in childhood (if at all), and if severe, the prognosis is unfavorable. Retardation of development, both mental and physical, is common.

The degree of anemia is variable, but it may be profound (R.B.C. 1.0 million or less). The color index and volume index vary, but usually are about 1.0. There are many reticulocytes and polychromatophilic cells, and often many normoblasts. The resistance of the red cells to hypotonic salt solution is normal or increased. There are usually a leukocytosis and an increase in platelets. In severe cases monocytes containing phagocyted red cells can often be found.

Elliptical red cells, which occur as a rare familial trait in Caucasians, must be sharply differentiated. The cells do not "sickle," and the condition usually does not cause anemia or impair the health. Such cells occur in cases of Mediterranean anemia (p. 453).

Table 50

DIFFERENTIATION OF THE ANEMIAS BY THE BLOOD PICTURE

	Hemoglobin	*Red Cells*	*Color Index*	*Volume Index*	*Anisocytosis*	*Poikilocytosis*	*Macrocytes*	*Microcytes*	*Color of Cells*	*Polychromatophilia*	*Reticulocytes*	*Nucleated Red Cells*	*Icterus Index*	*Urobilinuria*	*Leukocyte Count*	*Platelets*	*Gastric Acidity*	*Response to Iron*	*Response to Liver*
Acute posthemorrhagic	50–80	3.0–4.5	N-L	N-L	+	0	0	±	N-P	+	+	±	N	0	H	H	N	+	0
Chronic posthemorrhagic	20–60	2.0–4.0	L	L	+[2]	+	±	+	P	+	+	±	L N	0	N H	N-H	N	+	0
Idiopathic hypochromic	20–60	3.5–4.5	L	L	+	±	±	+[2]	P	0	N	0	L	0	N-L	N	0	+[2]	0
Hookworm	30–70	2.0–4.5	L	L	+	±	0	+	P	0	N	0	N-L	0	V	N	V	+	0
Cancer of stomach	20–80	2.0–4.5	L	L	+	±	0	+	P	±	V	0	N	0	N-H	N	0	+	0
Chlorosis	20–60	3.5–4.5	L	L	±	0	0	+	P	0	0	0	L	0	N	N	N	+[2]	0
Chronic infection, severe	30–70	2.5–4.5	L	L	+	0	0	+	P	0	0	0	N-L	0	V	N-L	N	0	0
Malaria	20–70	1.0–4.0	N	N	+	±	±	±	N	+	N-+	0	H	+	L	V	N	0	0
Pernicious, active stage	30–70	1.0–3.0	H	H	+[3]	+	+[2]	+	D	+	+	+	H	+	L	L	0	0	+[3]
Sprue: (1) macrocytic	30–70	2.0–3.5	H	H	+[2]	+	+[2]	+	D	±	N	±	N	±	L	V	N-0	0	+[2]
(2) hypochromic	30–70	3.0–4.5	L	L	+	+	+	+	P	0	N	0	N	0	L	V	N	+	0
Aplastic	30–60	1.5–3.5	N	N	±	±	0	0	N	0	0	0	N	0	L	L	N	0	0
Erythroblastic	30–80	1.5–4.5	N-L	N	+	±	±	±	N	+	+[2]	+[3]	N	0	H	H	N	0	0
Hemolytic	20–80	1.0–4.0	N	N	+	0	±	±	N	+	+	±	H	+	N-H	H	N	0	0
Familial hemolytic jaundice	30–70	1.5–4.0	N-H	N-H	+	0	0	+[3]	D	+[3]	+[3]	±	H	+[3]	N	N	N	0	0
Sickle cell	20–90	1.5–4.5	N	N	+[2]	+[2]	+	+	N-D	+[2]	+[2]	+[2]	H	+	H	N-H	N	0	0
Lead poisoning	50–90	3.0–5.0	N-L	N-L	±	0	0	±	N-P	+[2]	+[2]	+	N-H	±	H	N	N	0	0
Banti's disease	25–50	2.0–3.5	L	L	±	+	0	+	P	0	0	0	N	0	L	L	V	0	0
Thrombopenic purpura	50–90	2.5–5.0	N	N	0	0	0	0	N	±	0	0	N	0	V	L-0	N	0	0
Acute leukemia	20–60	1.0–3.0	N	N	+	±	±	+	N	+	+	+[2]	N	0	V	L-0	N	0	0

The data apply to average cases in patients in whom the disease is well marked but not of maximu severity. There are numerous exceptions and variations (see text).

D = Dark.
H = High or increased.
L = Low or decreased.
N = Normal or not characteristically altered.
P = Pale.
V = Variable.
\+ = Present or increased.
± = Inconstant or sparse.
0 = Absent or sparse.

Hemolytic Jaundice. Two types have been described: (1) a congenital familial form, and (2) an acquired form, occurring later in life, and without a familial history.

Familial Hemolytic Jaundice. This depends upon a constitutional anomaly which is transmitted by either sex as a dominant Mendelian characteristic. The disease usually becomes manifest during the second or third decade, and is characterized by recurring attacks ("crises") of hemolytic anemia, with the usual symptoms of acute hemolysis and outspoken jaundice. The serum bilirubin may be increased to from 10 to 50 times the normal. In the intervals there is partial recovery, but some anemia and jaundice persist. The acute attacks may be precipitated by an acute infection or other associated disease, but often no exciting factor can be found. Gall-stones develop in about half the cases, and gall-stone colic and obstructive jaundice may mask the underlying disease. The spleen is regularly enlarged. The bone marrow shows a marked hyperplasia which is usually normoblastic. Extramedullary areas of hyperplasia may occur. The disease causes marked disability, but is rarely directly fatal.

The degree of *anemia* is usually slight or moderate, but may be marked. The average red-cell count is from 3.0 to 3.5 million. The color index and volume index (and mean corpuscular volume) are normal or slightly increased. The distinctive features are: (1) The diameter of the red cells is diminished, but the mean volume is not significantly altered; hence the thickness must be increased, so that the cells are more globular than normal. Naegeli (1919) termed them "spherocytes," and regarded their formation as a manifestation of the constitutional anomaly underlying the disease. Krumbhaar has called the disease "spherocytic icterus." (2) The resistance to hypotonic salt solution is diminished. Hemolysis usually begins in concentrations from 0.5 to 0.6 per cent, rarely even 0.8 per cent (instead of 0.44 per cent), and may be complete at 0.4 to 0.48 per cent (instead of 0.34 per cent). Not infrequently, however, the divergence from normal is relatively slight. (3) Reticulocytes are much increased, often to 10 or 20 per cent, rarely to 50 per cent and more. Other evidences of regeneration are present, including frequently a few normoblasts.

Splenectomy stops the rapid cell destruction and usually effects a permanent clinical cure, although the abnormal shape and diminished resistance of the cells persist in some degree. Relapses have occurred, and in some cases have been associated with hyperplasia of accessory spleens.

Not infrequently examination of the relatives of a patient reveals *latent cases*, with a slightly diminished resistance of the red cells to hypotonic salt solution as the only manifestation of the anomaly.

Chronic Familial Jaundice. Dameshek (1941) has described a familial form of chronic mild jaundice, with an indirect van den Bergh reaction, but without evidence of increased blood destruction and attributed to a constitutional defect of the liver which interferes with the conversion and excretion of bilirubin. These persons showed no anemia, no splenomegaly, no spherocytosis, no reticulocytosis, normal fragility, normal or low urobilinogen excretion in the feces, and normal marrow smears. As far as observed there was no tendency to progressive liver disease, and except for delayed bilirubin excretion the usual tests for liver function gave normal values.

Acquired Hemolytic Jaundice. As usually defined, this differs fundamentally from the familial type only in the late onset and apparent lack of hereditary factors. It is usually attributed to some infection or other organic disease (which is not always demonstrable). Clinically the disease is described as usually more severe than the familial type, the anemia more profound (R.B.C. averages 2.0, minimum 0.5 million), and a fatal outcome common. Splenectomy is less regularly effective. The globular shape and diminished resistance of the red cells are often less clear-cut. As these features constitute the only decisive characteristics by which the condition can be differentiated from ordinary acute hemolytic anemias, many have questioned its existence as a disease entity.

Dameshek (Medicine, 1940) has reported producing in animals, by injections of hemolytic serum, an anemia which closely resembles acquired hemolytic jaundice in man, including the presence of spherocytosis and increased fragility of the red cells. He has pointed out the frequent occurrence in man of spherocytes in severe hemolytic anemias of both known and unknown origin, and believes no distinction can be drawn between acute hemolytic anemia and acquired hemolytic jaundice. Spherocytosis would therefore not indicate a constitutional anomaly of the bone marrow, but would result from injury to the mature red cells in the circulation, presumably by some lytic substance in the plasma. Hemolysins have been reported in the serum of a few cases of hemolytic anemia in man, but as yet little is known about them. Acute hemolytic anemia has been observed in a few patients whose blood contained "cold agglutinins."

Congenital Erythroblastic Anemia (Erythroblastosis Foetalis). This is a congenital, familial (but not directly hereditary) disease which may appear in three distinct clinical types, probably different stages or different degrees of severity of the same process (Diamond, Blackfan, and Batty, 1933). Successive children in the same family are often affected and may show different types of the disease. All types have the following characteristics in common: (1) A severe hypochromic type of anemia (R.B.C. often 1.0 million or less). (2) Extraordinary numbers of circulating erythroblasts (up to 100,000 per cu. mm.) of every stage of maturity. (3) Extreme hyperplasia of erythropoietic tissue, both intramedullary and extramedullary, in the spleen, liver, and many other organs. (4) Great enlargement of the spleen and liver. (5) A leukocytosis with many immature cells or rarely a leukopenia and thrombocytopenia. (6) Often a bright golden-yellow vernix caseosa and amniotic fluid.

Hydrops Foetalis. In this type the infant is stillborn or dies within a few hours. There is marked generalized edema of the placenta and fetal tissues, cardiac dilatation, dyspnea, and cyanosis.

Icterus Gravis Neonatorum. There is jaundice, which may be present at birth, or may appear during the first 12 to 48 hours. It increases rapidly to a deep orange-brown color. Petechiae may occur. The tissues are deeply icteric, including in some cases the basal ganglia ("Kernicterus"), and there are extensive deposits of iron pigment. The disease, if untreated, is often fatal within the first week, but it can usually be cured by repeated transfusions. Rh-negative blood should be given, or if this is not obtainable washed red cells (*not* whole blood) from the mother may be used. In severe cases it has been recommended that a massive transfusion be administered while simultaneously withdrawing blood from another vein in order largely to replace the original blood of the infant with that of a normal Rh-negative donor. Recovery, if it occurs, is complete and permanent, except for irremediable injury to the brain in some cases.

Congenital Anemia of the Newborn. This is practically identical with the preceding type except for lesser severity and the absence of jaundice. It is distinguishable from the simple hypochromic anemias chiefly by the erythroblastosis and splenomegaly.

It has been shown that in over 90 per cent of these cases there is an incompatibility between the mother and fetus with respect to the Rh antigen (see p. 408). As a rule the mother has been Rh-negative and the fetus Rh-positive, inheriting this characteristic from an Rh-positive father. It is believed that Rh antigen from the fetus enters the

maternal circulation and stimulates the formation of anti-Rh antibodies which in turn get into the fetal circulation and injure the red blood cells and other tissues. In rare instances other types of incompatibility seem to be involved. Erythroblastosis occurs in only a small proportion of those infants in whom it might be anticipated because of such an Rh incompatibility (about 1 : 15). Erythroblastosis is much more likely to occur if an Rh-negative mother has previously received an injection of Rh-positive blood. With each successive pregnancy with an Rh-positive fetus the tendency to erythroblastosis is increased, and if erythroblastosis has appeared in one infant, there is virtually no possibility that a subsequent pregnancy (with an Rh-positive fetus) will yield a healthy, living infant. It is manifestly essential that every Rh-negative woman should be transfused *only* with Rh-negative blood. If the father happens to be heterozygous, however, and carries one rh (negative) gene, there is a 50 per cent chance for the conception of an Rh-negative fetus, which will not develop erythroblastosis.

Mediterranean Anemia (Erythroblastic Anemia of Cooley, Thalassemia). This is a congenital familial constitutional anomaly largely limited to eastern Mediterranean races, including Italians. The severe form, seen in children, is characterized by: (1) a hypochromic microcytic anemia which becomes severe (red cells 1.7 million, Hb. 10 per cent); (2) marked anisocytosis and a bizarre type of poikilocytosis with thin cells which in fresh preparations are bowl-shaped and in stained films appear as target cells; oval cells are numerous in some cases, but sickling is stated not to occur;* (3) a marked erythroblastosis with normoblasts and macroblasts (often 100 or more per 100 W.B.C.); (4) increased resistance to hemolysis in hypotonic salt solution, some cells often remaining intact in 0.1 per cent saline and even in distilled water; (5) a leukocytosis, often with myelocytes and myeloblasts in the circulating blood; (6) mild jaundice and hyperbilirubinemia; (7) enlargement of the spleen and liver; and (8) marked generalized hyperplasia of the erythropoietic tissue, leading to peculiar characteristic changes in the bones, especially the long bones and skull. The cortex is thinned, and the medullary portion becomes widened and porous, so that in roentgenograms the trabeculae stand out like fine, sharp spines. The thickening of the bone may be so great that it gives the patient a mongoloid physiognomy with high, bulging forehead and prominent malar eminences. (Similar changes, usually less marked, may occur in severe cases of sickle cell anemia and familial hemolytic jaundice.)

The anemia is not noted at birth but becomes evident usually within the first year or two. The disease runs a progressive course without remissions and is uninfluenced by treatment; it is usually fatal within the first ten years, sometimes within a year. Splenectomy has proved useless, and is followed by a great increase in the erythroblastosis (up to 1500 nucleated red cells per 100 W.B.C.).

A mild anemia, qualitatively similar but often symptomless, has been noted in adults as well as children, particularly in parents, siblings, and other relatives of patients with the severe form of the disease. The alterations in the blood, particularly those described in (1), (2), and (4) above, are commonly observed in slighter degree, but erythroblastosis and bone changes demonstrable in roentgenograms are rarely present. Some cases intermediate in severity have been de-

*We have observed characteristic sickling in one such case, an Italian girl who showed no sign of negro ancestry.

scribed. It has been suggested that the severe form of the disease depends upon the inheritance of a recessive character (a defect which must be derived from both parents), and that the mild cases signify heterozygous carriers of the defect in a (relatively) latent form.

MISCELLANEOUS CONDITIONS

Banti's Disease. Banti's disease is a syndrome of unknown cause, occurring chiefly in young adults, and characterized by the gradual development of a hypochromic anemia, associated with splenomegaly, progressive weakness and emaciation, a tendency to gastric hemorrhages, and a terminal atrophic cirrhosis of the liver, with ascites and jaundice. The anemia is usually moderate (R.B.C. 3.0 million, Hb. 50 per cent), but evidences of red-cell regeneration are scanty. At times there are evidences of increased red-cell destruction. The fragility of the red cells is normal. There are usually a neutrophilic leukopenia and a moderate reduction in platelets. Splenectomy in the early stages of the disease may be curative, or at least temporarily beneficial. The advisability of this procedure has been questioned in patients with normal platelets because of the frequency of postoperative thrombocytosis and thrombosis in this group.

The cases commonly included under this heading constitute a heterogeneous group, and many investigators deny the existence of Banti's disease as a definite entity. Similar pathologic changes may follow occlusion of the splenic or portal veins from various causes. The condition should be regarded as a clinical syndrome rather than as a distinct disease.

Anemias in Young Children. Anemias in young children, regardless of their cause, often differ from those in adults in the type of cellular response. Immature red cells are more numerous, particularly normoblasts, macroblasts, and even megaloblasts. There is more regularly a leukocytosis (or lymphocytosis), which may be marked and accompanied by many immature leukocytes. There is often enlargement of the spleen, liver, and lymph nodes, due to erythroblastic hyperplasia in these organs. The *anaemia pseudoleukaemica infantum* of von Jaksch represents in marked degree such an infantile response to anemia resulting from a variety of infectious diseases and metabolic or nutritional disturbances.

POLYCYTHEMIA

By a polycythemia, or more precisely an *erythrocytosis*, is meant an increase above the normal in the number of red cells per cu. mm. of blood.

In a *relative polycythemia* there is no increase in the total number of red cells in the body. It is seen chiefly as a transient phenomenon associated with dehydration, and is a rough measure of its severity. A local erythrocytosis may occur as a result of local stasis, whether due to chilling and acrocyanosis, or to the use of a tourniquet.

In an *absolute polycythemia* there is an increase in the blood volume as well as in the red-cell count. There is a pathologic increase in the total number of red cells in the body. This occurs (1) in the specific disease erythremia (polycythaemia vera), and (2) as a compensatory response to anoxemia in the following conditions (erythrocytoses, secondary polycythemias):

1. In normal individuals at high altitudes. Within an hour or two after a sudden ascent (as in aviation) there may be a rise of 500,000 red cells per cu. mm., due to an outpouring of cells from the spleen and other reservoirs, which disappears promptly after descent to normal levels. With a sojourn at high altitudes, in some individuals there is an increased production of red cells, with a transient rise in reticulocytes, which is stimulated by the low O tension. The red-cell count may reach 8 million or more. In rare instances symptoms somewhat similar to those in severe erythremia may appear (chronic mountain sickness, Monge), which, however, are usually relieved by removal to lower altitudes.

2. In chronic myocardial insufficiency with cyanosis, as in some cases of mitral stenosis and congenital pulmonary stenosis.

3. In emphysema, in diffuse pulmonary fibrosis from any cause, and in sclerosis of the pulmonary arteries (*Ayerza's disease*).

4. After mild chronic poisoning with a variety of blood poisons, such as lead, carbon monoxide, cobalt, and coal-tar products.

Erythremia (Polycythaemia Vera, Rubra). This is a chronic disease of unknown cause, characterized by an erythrocytosis with an increase in total blood volume, by a peculiar intense flushing of the skin and mucous membranes, by enlargement of the spleen, and by a normoblastic hyperplasia of the erythropoietic tissues, rarely in extramedullary areas. It affects chiefly adults over 50 years of age. It is characterized clinically by an insidious onset and a protracted chronic course, with weakness, headaches, vertigo, tinnitus, paresthesias, nervous irritability, and mild mental disturbances. The skin, especially of the face and extremities, acquires an intense, mottled, brick-red color, and the mucous membranes a deep purplish-red or plum color, due to marked dilatation of the superficial capillaries together with slowing of the local circulation. Hemorrhages and thromboses are common. Arteriosclerotic changes are often marked, both in the peripheral and cerebral vessels. There is often hypertension, and resulting myocardial insufficiency. Occasionally cirrhosis of the liver develops, or arteriolosclerosis of the kidney and renal insufficiency. The basal metabolic rate is increased in about half the cases.

The blood at some stage of the disease usually shows a red-cell count of 8 million or more, rarely even 12 million, with the volume of packed red cells 80 or even 90 per cent. Higher counts would be physically impossible unless the mean corpuscular volume were extremely low. However, the count is not invariably or constantly so high. In undoubted cases it may be between 5 and 6 million. The blood volume is always increased and may be double or even treble the normal, but a marked increase may occur in secondary erythrocytoses. The hemoglobin is increased to a relatively less degree; usually 19 to 24 Gm., 130 to 160 per cent. The viscosity is from two to five times the normal. The individual red cells are somewhat small and pale, they show slight anisocytosis, and usually a moderate increase in reticulocytes and other immature forms. Platelets are increased. There is a moderate leukocytosis, which may become marked and associated with a slight myelocytosis. Death is due most frequently to cardiovascular renal disease or other associated pathologic conditions, occasionally to hemorrhage or thrombosis. In a substantial number of those who escape such complications the terminal stage is

marked by the clinical and hematologic manifestations of a chronic myelogenous leukemia or, more rarely, of an aplastic anemia.

Symptomatic relief with restoration of a normal blood count (but usually not a normal blood picture) may follow repeated venesection, radiation of the long bones, or the *cautious* administration of phenylhydrazin or radio-active phosphorus.

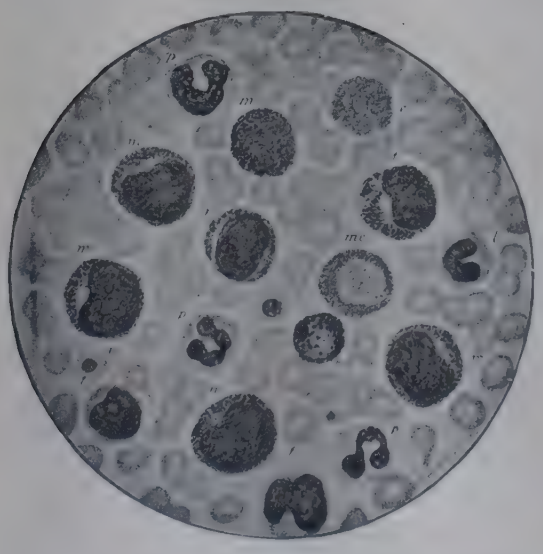
The resemblance of the symptoms and changes in the blood to those of mountain sickness has suggested that there is an anoxemia of the tissues in erythremia. However, no mechanism for the production of such an anoxemia has yet been demonstrated, except that thickening of the walls of the capillaries in the bone marrow has been described. Some regard the process as a malignant hyperplasia, analogous to leukemia.

Diseases Involving Primarily the Leukocytes

Leukemias

Leukemia is a disease characterized by an abnormal proliferation of the leukopoietic tissue, and by the appearance in the circulating blood of immature leukocytes which are not present in normal blood. There is usually a marked increase in the number of circulating leukocytes, but there is no direct relation between the height of the leukocyte count and the degree or activity of the hyperplasia.

There are three distinct types of leukemia, termed myelogenous, lymphatic, or monocytic, depending upon which of the leukopoietic tissues is involved. Clinically, acute and chronic forms of each type may be differentiated, depending upon the mode of onset and the duration of the disease. Cases in patients living less than four months are arbitrarily classed as acute, but every gradation occurs. The cause of the disease is unknown. There are many analogies to malignant neoplasms.



Chronic myelogenous leukemia. (*m*) Myelocyte. (*p*) Polymorphonuclear. (*b*) Mast cell. (*n*) Normoblast. (Cabot.)

Chronic Myelogenous Leukemia. Chronic myelogenous leukemia, the most common type, is a disease of adults, characterized clinically by an insidious onset, with symptoms referable to the enlarged spleen, and progressive weakness, emaciation, and anemia. Symptoms of myocardial insufficiency and digestive disturbances frequently occur, and occasionally hemorrhages, pruritus, priapism, and pain and tenderness in the long bones. The spleen is huge, often reaching to the right anterior superior spine. The liver is usually much enlarged, the lymph nodes rarely.

The *blood* shows a total leukocyte count which is usually between 100,000 and 500,000 per cu. mm., but rarely it may reach 1.0 or 1.5 million. Occasionally it is within the range of an ordinary leukocytosis. As a rule the bulk of the leukocytes are polymorphonuclear neutrophils and neutrophilic metamyelocytes (30 to 65 per cent). Neutrophilic myelocytes are always present (5 to 70 per cent, usually 20

to 50 per cent). Eosinophils and basophils are increased, and occasionally either type of cell may be markedly increased (up to 40 per cent). The corresponding types of myelocytes are present, and there may be an occasional primitive myelocyte or myeloblast. Pathologic leukocytes of bizarre appearance are often found. The lymphocytes and monocytes are relatively reduced.

The platelets are much increased.

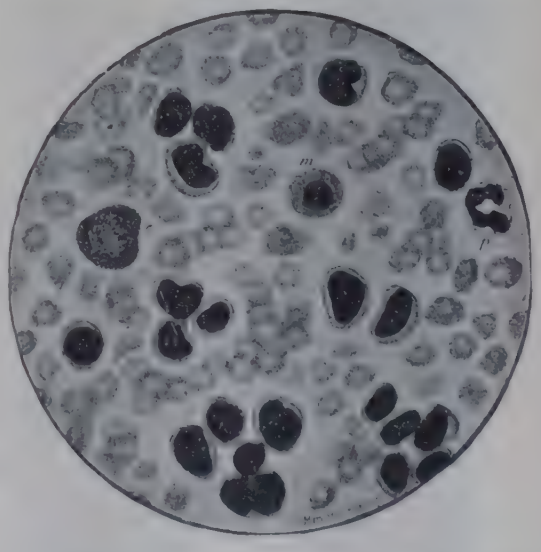
The red-cell count is reduced, and is usually from 1.0 to 3.0 million. The mean corpuscular volume and hemoglobin content are about normal. Immature red cells are increased, and normoblasts are constantly present, often in fairly large number; rarely, a few megaloblasts. The anemia is myelophthisic in type, resulting in part from the crowding out of the erythroblasts by the hyperplastic leukopoietic tissue. There may be signs of increased hemolysis.

Films of the bone marrow show a relative increase in the immature types of granular leukocytes and may closely resemble films of the circulating blood. They are rarely needed for diagnosis and are often less helpful than in other types of leukemia because the changes are less distinctive.

Remissions may occur, either spontaneously, possibly in association with an acute infection, or after radiation or other effective therapy. During a remission, or in the incipient stages of the disease, the total leukocyte count may be normal (*aleukemic myelosis*), and the diagnosis may be missed without a careful differential count. This almost always reveals myelocytes (5 to 30 per cent) and normoblasts.

The disease often terminates with an acute exacerbation, during which the blood takes on many of the characteristics of an acute leukemia (described below). This is ushered in by fever, an abrupt aggravation of the clinical symptoms, a rapid fall in red-cell count and hemoglobin, often thrombocytopenia and purpura, and the appearance of many primitive myelocytes and myeloblasts. There is usually no hiatus leukaemicus (p. 460). The total leukocyte count may rise or fall.

Difficulties in diagnosis occur chiefly in the hyperleukocytoses due to infection, hemorrhage, etc., and in focal lesions of the bone marrow when these are associated with a stimulation myelocytosis. Further study is required to determine whether a reliable differentiation can be made by a study of films of the bone marrow.



Chronic lymphatic leukemia. (*p*) Polymorphonuclear. (*m*) Megaloblast. (*e*) Eosinophil. Twenty-one lymphocytes in this field. (Cabot.)

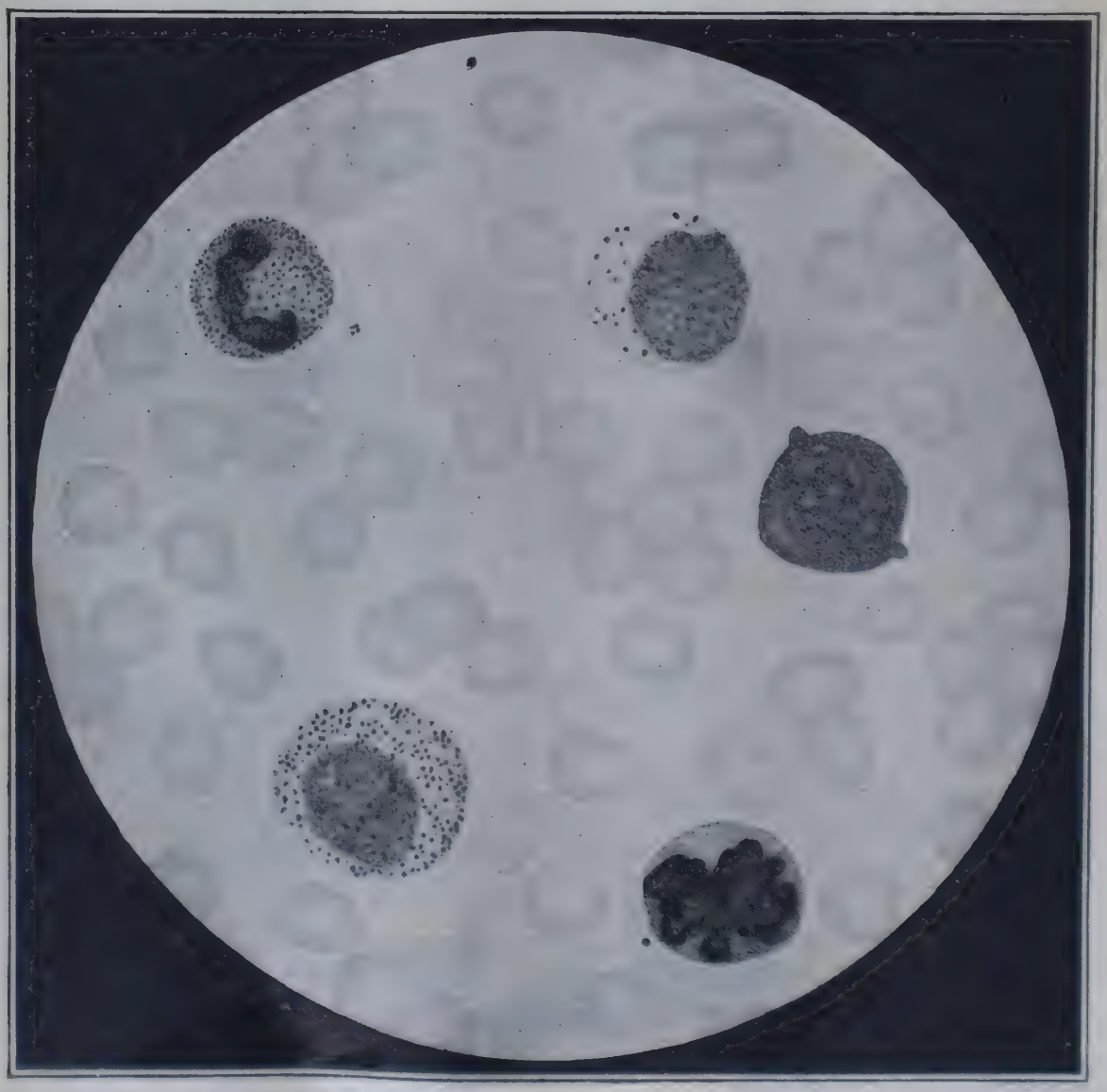
Chronic Lymphatic Leukemia. This is also a disease of adults, which resembles the preceding type in its symptoms and clinical course except that there is a more marked generalized enlargement of the lymph nodes, tonsils, and other aggregations of lymphoid tissue. Local infiltrations in the skin and mucous membranes are common. Splenomegaly is less marked.

The *blood* shows an increase in the total leukocyte count which may range from 10,000 to 2.5 million, but in most cases is from 100,000 to 200,000, somewhat lower

than in chronic myelogenous leukemia. Counts under 50,000 are not uncommon. From 90 to 99 per cent of the cells are lymphocytes, practically all small cells with nuclei of mature type containing dense chromatin masses. The cytoplasm is often scanty, and azure granules are sparse or absent. There are usually a few lymphoblasts, some of which may contain lobulated or indented nuclei (*Rieder cells*). Granulocytes may be absolutely, as well as relatively, reduced. The platelets become diminished. In the later stages an aplastic type of anemia develops. Normoblasts are rare.

Cases occur, anatomically and clinically identical, except that the total leukocyte count is normal. They usually show a relative lymphocytosis. The blood may become frankly leukemic at any time, or death may ensue before this occurs (*aleukemic lymphadenosis*, "aleukemic" lymphatic leukemia).

Diagnostic difficulties arise chiefly in infections with a high lymphocytosis, such as infectious mononucleosis and whooping cough; and in diseases associated with generalized enlargement of the lymph nodes and spleen (Hodgkin's disease, lymphosarcoma). Cases of lymphatic leukemia with a relatively low total leukocyte count are difficult to recognize by an examination of the blood alone, because the differences between mature



Acute myelogenous leukemia. (W.B.C. 13,000.) Actual field. (*Right*) Myeloblast. (*Upper right*) Promyelocyte. (*Lower left*) Myelocyte. (*Lower right*) Myeloblast in mitosis.

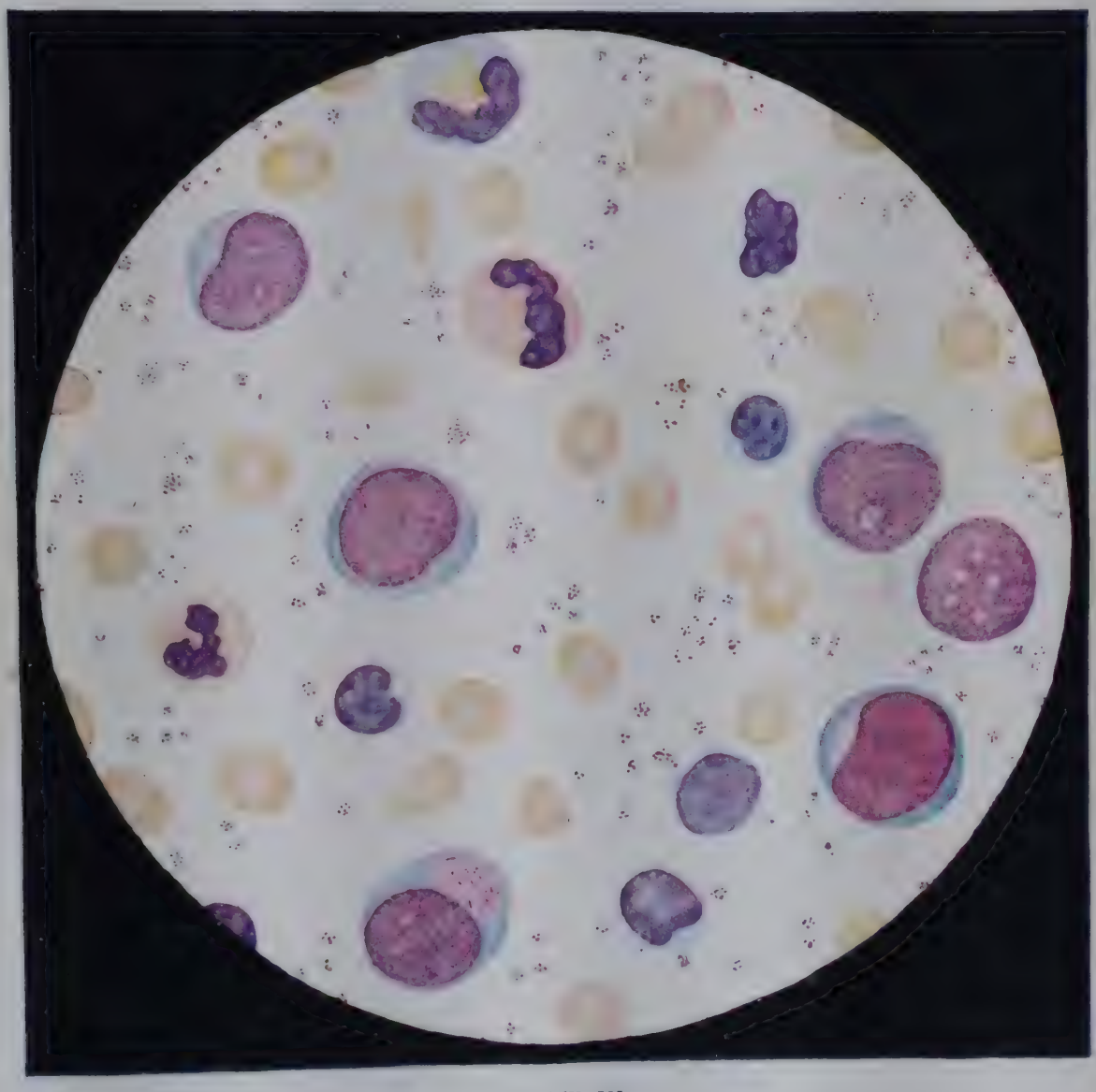


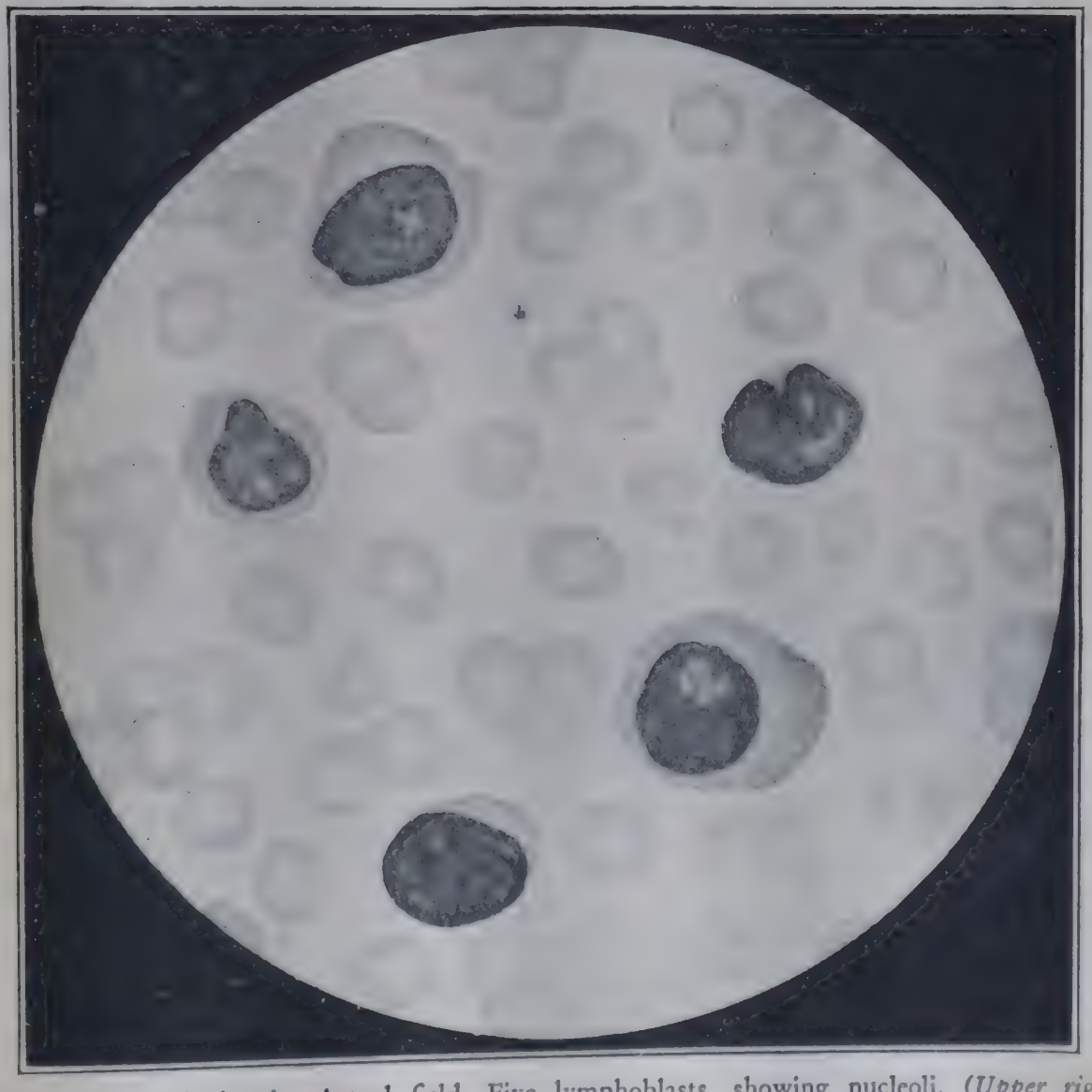
PLATE III

Acute leukopenic myelogenous leukemia. (W.B.C. 3000.) Film from buffy coat (see text). Actual field. Five myeloblasts, several showing nucleoli. (*Lower left*) Promyelocyte. Three nonsegmented neutrophils, practically destitute of granules (toxic-degenerative changes). (*Lower right*) Two megakaryocyte nuclei with shreds of platelet material attached.

and immature lymphocytes are less conspicuous than in the case of the granulocytes and because lymphoblasts appear in the blood more frequently than myelocytes in conditions other than leukemia. Films from the bone marrow usually show a marked increase in lymphocytes.

Acute Leukemia. This may be myeloid, lymphoid, or monocytic in type. Clinically the three types are practically indistinguishable. The disease occurs chiefly in children and young adults. In about half the cases of acute leukemia the onset is preceded by an acute infection, or there is a history of repeated chronic infections. In some cases the sequence is so direct that one can scarcely escape the conclusion that the leukemia represents a perverted (irreversible) response to the stimulus of the infection. Clinically the disease cannot always be differentiated sharply from infections associated with a (reversible) "leukemoid reaction." Diagnosis may be possible only by biopsy of the marrow or by observing the ultimate outcome. However, some individual susceptibility or defect of the marrow must be assumed. The infection can at most be regarded as only a precipitating cause of the leukemia.

In some cases there is a preliminary period of vague ill health, weakness, and pallor. Usually the onset of symptoms is fairly abrupt, and may be fulminant, with fever, weakness, prostration, and often sore throat, followed quickly by an ulcerative stomatitis and gingivitis, and a hemorrhagic diathesis with purpura and oozing from the gums and



Acute lymphatic leukemia. Actual field. Five lymphoblasts, showing nucleoli. (*Upper right*) Rieder cell.

other mucous membranes, and a rapidly developing anemia. Clinically it resembles an acute septic infection. There is progressive enlargement of the spleen. Death usually occurs within two to four months, and may occur within two weeks. Remissions, however, may occur. The leukemic infiltration in the marrow undoubtedly precedes the appearance of symptoms by a substantial period in these acute cases.

The leukocyte count in the early stages may be normal or moderately increased. There may be a well-marked leukopenia (*"aleukemic leukemia"* or, better, leukopenic leukemia). As a rule there is a progressive increase in the count, which often exceeds 100,000 within a few weeks. The characteristic feature is the appearance in the blood of increasing numbers of primitive leukocytes. The platelets are much reduced.

In the leukopenic cases the study of the cells is greatly facilitated by drawing blood from a vein into one-third its volume of 1.5 per cent sodium citrate solution, centrifuging and making films from the buffy coat (see Plate III). Examination of a bone-marrow film is of great practical diagnostic value in these cases. The distinctive primitive cells are usually present in great numbers and are unmistakable.

Acute Myelogenous Leukemia. In acute myelogenous leukemia, the commonest type, the primitive cells are promyelocytes or myeloblasts. There is not a gradual transformation or transition from normal leukocytes, through myelocytes of varying stages of immaturity, to myeloblasts. In the early stages of the disease the primitive cells appear, at first in small numbers, as intruders in a leukocyte population which is essentially normal. There is a "hiatus leukaemicus" (Naegeli). As the myeloblasts increase in number the mature leukocytes are rapidly crowded out, and may entirely disappear. The exact type of primitive cell varies considerably in different patients, but is usually quite uniform and constant in the same case. Careful search usually reveals a few cells which are somewhat more mature (myelocytes), a finding which may be of great assistance in identifying the type cell. In some cases the primitive cells are small and almost indistinguishable from small lymphocytes (micromyeloblasts). Normoblasts are regularly present, occasionally megaloblasts.

Acute Lymphatic Leukemia. This affection is very rare except in children. The predominant cells are lymphoblasts. Cases with marked leukopenia occur. Progressive enlargement of the lymph nodes usually occurs, and in some cases the nodes form large tumor-like masses. Rarely the lymphoid hyperplasia may be limited largely to the marrow and the deeper nodes.

Monocytic Leukemia. Monocytic leukemia usually runs an *acute* course. It occurs in individuals of any age. Clinically this type is characterized by marked hyperplasia (and ulceration) of the gums, which may become so great as to engulf the teeth; by the absence of marked lymph-node enlargement, except often of the cervical nodes; and often by excessive bleeding, and a rapidly fatal course. The leukocyte count ranges usually from 20,000 to 400,000. The predominant cells (20 to 90 per cent) are monocytes and their precursors. Their differentiation from early myelocytes is sometimes difficult. The most characteristic features are the infolding of the nuclear membrane so as to form coarse convolutions and the delicate structure of the chromatin network, resembling fine lace. Motility and phagocytic activity in fresh cover-slip films is decisive evidence that they are monocytes. Myelocytes and myeloblasts are usually present in small numbers, and in some cases they are numerous, and give the appearance of a "mixed leukemia." This has led Naegeli and others to question the existence of monocytic leukemia as a distinct disease. With this possible exception, there is no evidence of the occurrence of true "mixed leukemia," with simultaneous involvement of two or more of the leukopoietic tissues, nor of transformations from one type of leukemia to another. Downey distinguished between a Naegeli type characterized by the presence of many myelocytes and myeloblasts as well as monocytes which he believed arise in the bone marrow, and a Schilling type in which the cells are largely monocytes which are thought to arise more directly from reticuloendothelial cells.

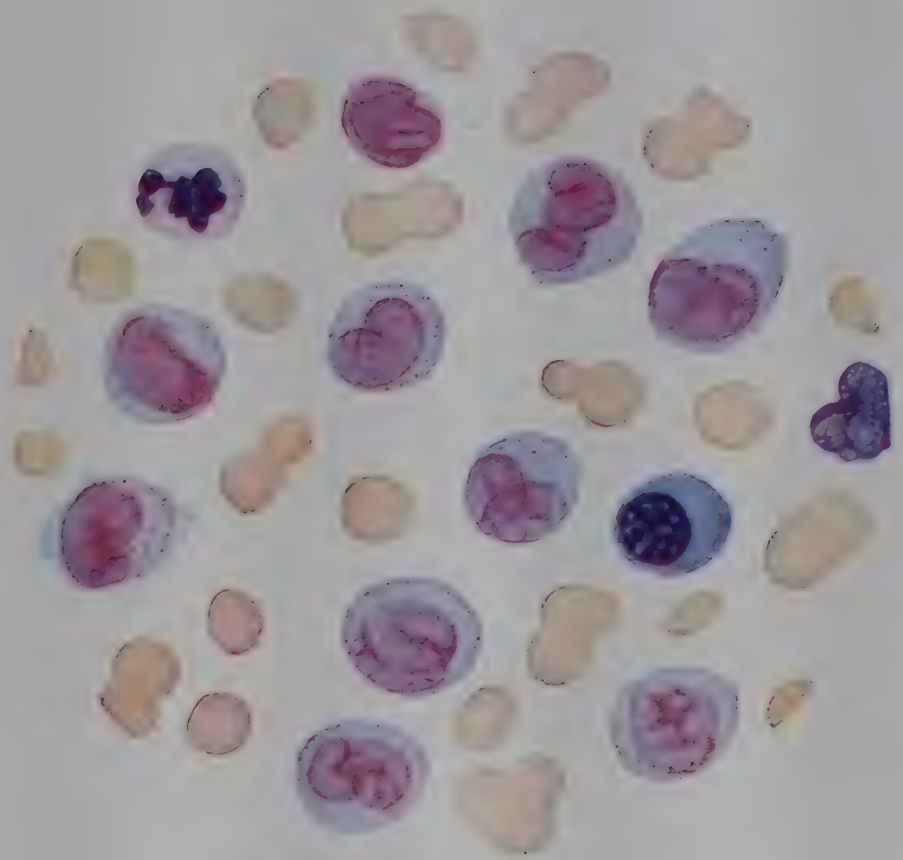


PLATE IV

Acute monocytic leukemia. Actual field. Eight of the cells are monocytes. They show a gray-blue cytoplasm with fine reddish lilac granulations, and nuclei of finely reticular structure with various types of infoldings and convolutions. (*Upper left*) A hypersegmented polymorphonuclear neutrophil. Below it, a monoblast with more basophilic nongranular cytoplasm and a more finely reticulated nucleus with several small nucleoli. Below this, a monocyte showing several pseudopods containing only clear cytosarc. (*Right center*) A plasma cell. (*Extreme right*) A smudged nucleus. Wilson's stain. (× 800.) (From *Bull. Johns Hopkins Hosp.*)

There is an extraordinarily widespread, diffuse hyperplasia of the reticulum cells in the tissues.

Cases with a normal leukocyte count or a leukopenia occur, but they usually show a relative monocytosis (*aleukemic reticulo-endotheliosis*).

SPECIAL TYPES OF LEUKEMIA

Chloroma. This is a rare type of leukemia in which localized, invasive, tumor-like growths of leukopoietic tissue occur. The growths are most often connected with the bones of the skull, especially the orbit, and may cause bony deformities and often protrusion of the eyeballs. They often cause intense pain or paralyses from pressure on neighboring structures. The tumor masses on section (in gross) usually have a green color. The changes in the blood and the clinical course are otherwise identical with acute or subacute myelogenous leukemia. All transitions to the ordinary type of leukemia occur.

Eosinophilic Leukemia and Mast-cell Leukemia. These terms are sometimes applied to cases of chronic myeloid leukemia which show an unusually large percentage of one of these types of cells. Aside from these cases, which are only minor variants of ordinary chronic myelogenous leukemia, there are records of a few cases in which enormous numbers of *mature eosinophils* were present, without other significant abnormality in the leukocytes. These cases ran the course of a subacute (rarely chronic) leukemia, and at autopsy showed leukemic infiltration of the tissues with eosinophils (Hay and Evans, 1929).

Plasma-cell Leukemia. This is a rare type of (lymphatic) leukemia in which many plasma cells are present in the blood, and in which the hemopoietic tissues are infiltrated with plasma cells at autopsy. Transitions occur to the localized plasmacytomas seen in multiple myelomas (Osgood, 1934).

Megakaryocytic Leukemia. A pathologic hyperplasia of the megakaryocytes, analogous to a leukemia, has been reported in a few cases.

Diseases Allied to or Resembling Leukemia

Pseudoleukemia is an inexact and undesirable term loosely applied to any condition superficially resembling lymphatic leukemia, but not showing leukemic changes in the blood.

Lymphoblastoma is a term applied to a group of diseases characterized by an abnormal hyperplasia of the lymphoid tissue, with enlargement of the spleen and lymph nodes. It includes lymphatic leukemia (both in the leukemic and aleukemic stages), lymphosarcoma, and Hodgkin's disease, but not the infectious granulomas or ordinary neoplasms.

Hodgkin's Disease (Malignant Granuloma). This is a chronic disease of unknown origin, possibly an infection, characterized by an infiltration of the lymphoid tissues and spleen (and occasionally the marrow and other organs containing reticulo-endothelial tissue) with a peculiar type of granulation tissue rich in epithelioid cells and giant cells (derived from monocytes, Sabin) and eosinophils, which proceeds to extensive fibrosis in the advanced stages. The process starts in some single aggregation of lymphoid tissue, most often in the upper posterior cervical or supraclavicular nodes on one side, or in the mediastinal nodes, less often in the deep abdominal nodes. It spreads first to the adjacent groups of nodes, and secondarily to more distant groups. The spleen usually becomes enlarged. The nodes remain discrete, but often form large masses which cause grave disturbances from pressure on neighboring structures. Bone lesions occur in 10 to 15 per cent of the cases. The lung may be invaded. Intense pruritus, which may precede evi-

dent lymph-node enlargement, is a characteristic clinical symptom, as (in some cases) are recurring paroxysms of high fever (*Pel-Ebstein*), prostration, emaciation, and progressive anemia, alternating with periods of normal temperature and relative well-being.

The *blood* is never leukemic. There may be a neutrophilic leukocytosis. In the early stages there may be an increase in monocytes and in platelets, and occasionally an eosinophilia, which is sometimes high (60 per cent or more of 100,000). There is no lymphocytosis, and in the late stages the lymphocytes may be reduced. A severe anemia gradually develops, which is somewhat hypochromic and microcytic in type. Biopsy is necessary for a positive diagnosis.

In the early stages deep radiation nearly always causes the glandular masses to disappear, with marked clinical improvement. Radiation soon loses its effectiveness, and death always follows, usually within two to five years, although a few patients have been reported to have survived 20 years.

Lymphosarcoma (Kundrat). Lymphosarcoma is a peculiar type of malignant tumor arising from lymphocytes. It starts locally, most often in the cervical or mediastinal nodes, occasionally in the abdomen, and spreads both by direct extension and by metastasis to neighboring groups of nodes. Late in the disease there may be metastases to other tissues, but the growth tends to ensheathe rather than invade the neighboring organs. Enlargement of the liver and spleen is rare except in those cases in which the involvement is primarily abdominal. Growth is often rapid, and the glands tend to coalesce and form large tumor masses. A practically complete temporary remission usually follows deep radiation.

As a rule the *blood* is not leukemic, and the lymphocytes are not increased. In a few cases, however, in the terminal stages there is an invasion of the blood stream by the pathologic cells (*leukosarcoma*). There is an anemia and usually a moderate neutrophilic leukocytosis. Biopsy is required for a positive diagnosis, but in the early stages differentiation from leukopenic lymphatic leukemia may be difficult.

Cases which appear to be rare variants of lymphosarcoma or Hodgkin's disease, based upon differences in the histologic changes in the tissues, have been described under the terms *reticulum-cell sarcoma, monocytoma, follicular lymphoblastoma,* and *Hodgkin's sarcoma.*

Follicular lymphoblastoma is characterized histologically by the formation of huge "giant" follicles in the lymphoid tissue. Clinically it resembles Hodgkin's disease, but often the splenomegaly is greater in degree and out of proportion to the enlargement of the peripheral lymph nodes. The disease at first usually responds well to radiation and it tends to run a more benign, protracted course than Hodgkin's disease, but it is eventually fatal.

Myeloma. Myeloma is a malignant neoplasm which is said to arise from any of the parenchymal cells of the marrow. In most if not all cases, however, it is composed of peculiar ("myeloma") cells which differ from all the cells of normal blood and marrow, and most closely resemble plasma cells, of which, according to some, they are precursors. The disease is focal but widespread (more rarely diffuse), and multiple nodules develop, usually in many bones. These may give rise to palpable swellings or to erosions

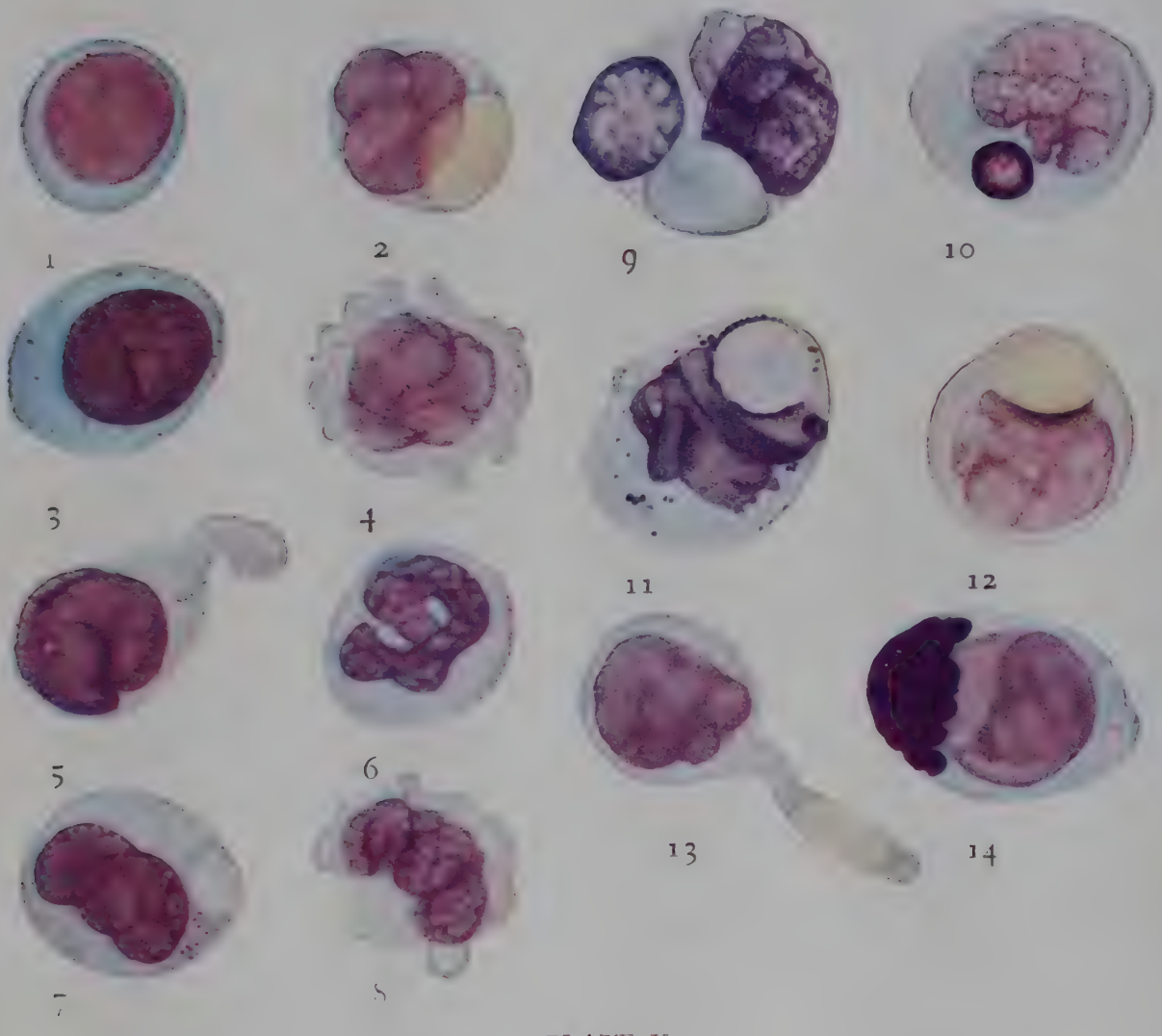


PLATE V

Cells from case of acute monocytic leukemia. (1 to 14) Monocytes. (1 and 3) Monoblasts.

often in the flat bones, particularly of the skull, which are eroded by localized yellowish nodules (*xanthomata*) of hyperplastic tissue arising from the periosteum or dura. Similar nodules may be found in the skin and serous membranes. Common secondary symptoms are exophthalmos and diabetes insipidus (from pressure of nodules on the hypothalamic region). There is also diffuse hyperplasia of reticulum cells in the marrow, liver, and spleen (although these organs are only slightly enlarged), and in the lungs and other organs. The early lesions contain large numbers of typical "foam" cells. In the late stage fibrosis occurs. Extensive pulmonary fibrosis may be the immediate cause of death.

The blood shows no characteristic changes. In late stages there may be a grave anemia of the myelophthisic type, and a thrombocytopenia with purpura. In some cases there has been a leukocytosis, with a moderate monocytosis. The blood cholesterol is often high, but has not been uniformly so.

Foam cells of the same type have been found in the spleen in some cases of *diabetes* and "nephrosis," in association with high blood fat and cholesterol.

Infectious Mononucleosis (Glandular Fever). This is a benign infectious disease of unknown origin which bears a superficial resemblance to acute leukemia, both in its clinical features and in the lymphocytosis which accompanies it. It occurs chiefly in children or young adults, both as sporadic cases and in epidemics. Clinically it is characterized by an acute onset with fever, usually sore throat, marked swelling of the cervical lymph nodes, a general glandular enlargement of variable degree, and often enlargement of the spleen. The cervical nodes may become huge. Occasionally there is a complicating Vincent's infection. Recovery practically always occurs within a few weeks, but the glandular enlargement and the lymphocytosis may persist for months.

The total leukocyte count is usually from 10,000 to 20,000, with a lymphocytosis of from 50 to 90 per cent. In some cases there is a normal total count, or there may be a leukopenia. The lymphocytosis may not reach its peak until after one or two weeks. The characteristic feature of the blood is the large number of *pathologic lymphocytes* present. These are mainly large cells with abundant, usually basophilic cytoplasm which shows a spongy or foamy texture and usually contains numerous azure granules. Most of the cells show a pale perinuclear zone. The nucleus is often indented or partly lobulated. It is usually mature in type, and shows a coarse chromatin network of thick strands and masses which may be quite dense, like plasma cells. Some typical plasma cells are often present. In some cases there are a few lymphoblasts, with nuclei showing a fine chromatin structure and nucleoli, and we have even seen cells in mitosis, but immature cells are not present in large numbers, as in acute lymphatic leukemia (see also Downey, 1922, 1935). The granulocytes are reduced in absolute number, and show toxic degenerative changes. The monocytes are diminished (or absent, Doan). The red cells and platelets are not altered, and there is no tendency to bleed. The lymph nodes show hyperplasia, with proliferation of the peculiar lymphocytes, which often obliterates the normal architecture of the node, but is less extensive and more "patchy" than in leukemia. The unusual leukocytic response is definitely due to the peculiar stimulus exerted by the infectious agent, and not to a constitutional abnormality of the patient.

The serum in most cases, after 7 to 10 days, shows an increase in *agglutinin for sheep red blood cells* (Paul and Bunnell, 1932).

Method. (1) A series of 12 small test tubes is set up. (2) In tubes 1 and 2 is placed 0.5 ml. of the serum to be tested, which has been inactivated by heating to 56° C. for 20 minutes. (3) To tubes 2 to 12 inclusive 0.5 ml. of salt solution is added. (4) A series of dilutions, from 1 : 2 to 1 : 2048, is prepared by transferring 0.5 ml. of the mixture in

tube 2 to tube 3 and mixing, and so successively to tube 12. (5) To each tube is added 0.5 ml. of a 2 per cent suspension of sheep corpuscles, washed as in preparation for a Wassermann test. (6) To each tube is added 1 ml. of salt solution. (7) The tubes are shaken and placed in a water bath at 38° C. for one hour, and in the icebox overnight. (8) The following morning they are shaken gently to resuspend the sedimented cells, and the highest dilution in which definite macroscopic agglutination occurs is noted; the degree of agglutination in each tube is recorded roughly.

In normal serum the titer usually does not exceed 1 : 8 (the first four tubes) (as recorded by Paul and Bunnell; the actual dilution is four times this figure). In infectious mononucleosis it usually rises to much higher figures (occasionally to 1 : 4000), although several examinations at intervals of a few days may be necessary to detect it. A rise in titer has been observed in individuals who have received injections of horse serum, but it is rare in other conditions. The titer is reduced (less than 1 : 4) in leukemia (Bernstein, 1934).

Recent work (Bailey and Raffel, 1935; Davidsohn, 1938) indicates that this agglutinin is not, as originally supposed, the heterophil agglutinin of Forssman. Three different anti-sheep cell agglutinins can be distinguished in human serum by appropriate absorption tests. (1) The Forssman type, that commonly found in normal serum, is absorbed by guinea-pig kidney but not by autoclaved ox cells. (2) The type occurring in infectious mononucleosis is absorbed by ox cells but not by guinea-pig kidney. (3) The type appearing after injections of horse serum is absorbed by both. In questionable cases the dependability of the test is increased by demonstrating that the agglutinin is absorbed as indicated in (2) above.

Acute Infectious Lymphocytosis. This term was applied by Smith to a benign contagious disease of unknown etiology observed thus far only in children and a few young adults. The illness is brief and often mild or symptomless. After an incubation period of two to three weeks there are usually manifestations of an infection of the upper respiratory passages, fever, and in the severer cases malaise, vomiting, headache, rarely signs of meningeal irritation, and abdominal pain which may be severe. The spleen and lymph nodes are not enlarged. There is a marked leukocytosis from 15,000 to usually about 40,000 (in one case 110,000), of which from 60 to 90 per cent are mature small lymphocytes. There are no lymphoblasts and no bizarre cells of the type seen in infectious mononucleosis. There is no increase in heterophil agglutinins. There is no anemia or thrombocytopenia. Sternal marrow obtained by puncture showed a moderate increase in small lymphocytes (about 40 per cent). All patients have recovered without complications or sequelae, but the lymphocytosis may last from three to seven weeks.

Agranulocytic Angina (Malignant Neutropenia). This is a clinical syndrome of unknown cause, occurring chiefly in women, characterized by an acute onset with fever, prostration, ulcerative stomatitis or pharyngitis, an extreme neutrophilic leukopenia, and usually a rapid progressive course and a fatal termination. Clinically the condition suggests a fulminant septic infection, but blood cultures are negative, or show organisms which can only be secondary or terminal invaders. The tissues do not show pyemic lesions, and there is a striking absence of leukocytic infiltration. Vincent's organisms are frequently (but not constantly) present in the mouth lesions.

The *blood* shows a marked (or rapidly progressive) leukopenia, primarily of the granulocytes, which may entirely disappear. The total count is usually under 2000 and may fall to 100. The red cells and platelets are usually unaltered, and bleeding rarely occurs. There is no stimulation of the lymphocytes such as occurs in infectious mononucleosis. If the patient survives a week or more, granulocytes may reappear, and not rarely

a few myelocytes and myeloblasts may be found, suggesting an acute leukopenic leukemia. The sternal marrow (biopsy) at the height of the disease often shows practically complete disappearance of granulocytes, without a proliferation of primitive cells such as is found in leukemia. In some cases the marrow has been highly cellular, with apparently an arrest of maturation at the myelocyte stage or earlier. In a few cases in which there is anemia and thrombocytopenia, differentiation from acute leukopenic leukemia may be difficult.

Recovery occasionally occurs spontaneously, or (possibly more often) after nucleotide injections. In such cases a normal blood picture may be restored, but often a lesser degree of granulocytopenia persists. Such patients are prone to recurring acute attacks, any one of which may prove fatal. Milder cases with less marked granulocytopenia occur and are probably more common than has been realized.

The most effective treatment appears to be administration of penicillin to ward off secondary infection and prolong life until recovery occurs and the normal defense mechanisms are restored.

In a few cases an extreme leukopenia has been recognized shortly before the onset of the fever and symptoms of acute illness. This, with other facts, suggests that in this condition damage to the marrow, or a constitutionally defective marrow, is the primary factor, and that infection occurs secondarily, as a result of lowered resistance due to lack of leukocytes. There is a striking tendency to the development of ulcerative or necrotic lesions of the mouth in other diseases associated with a lack of granulocytes (as in acute leukemia). That the marrow may be inherently defective is shown by the occurrence of individuals who show, over a period of years, either persistent or periodically recurring granulocytopenia, which may or may not be accompanied by active illness. Recently, however, emphasis has been placed on the use of *aminopyrine* and related drugs as a cause of the granulocytopenia. There is definite clinical and experimental evidence that aminopyrine tends to injure the marrow in this way. Such drugs should be rigidly withheld from patients with this tendency. However, the rarity of the condition, as contrasted with the widespread use of these drugs, indicates that individual predisposition (probably an allergic hypersensitiveness) plays a large part. Acetylsalicylic acid and the simple barbiturates appear to have relatively little of this effect.

Primary Splenic Neutropenia. This syndrome is characterized by a marked leukopenia with reduction of the granular leukocytes. As a rule there is no notable anemia or thrombocytopenia. There may be weakness, fever, and increased susceptibility to infections. The spleen is enlarged, and in supravitally stained films Wiseman and Doan (1942) reported finding widespread phagocytosis of granular leukocytes by the reticulo-endothelial cells. There were no evidences of hematopoiesis in the spleen, but the sternal bone marrow was hyperplastic. The condition was relieved by splenectomy.

Primary Splenic Panhematopenia. This term has been applied by Doan and Wright (1946) to a group of cases characterized by severe anemia and thrombocytopenia as well as a neutrophilic leukopenia. These cases showed pathologic changes in the spleen and bone marrow similar to those in primary splenic leukopenia, and splenectomy was equally beneficial. Both acute and chronic clinical types have been described. As an aid in differentiating these cases from the ordinary aregeneratory anemias Doan and Wright utilized an adrenalin test. Following subcutaneous injection of 0.5 to 1.0 ml. 1 : 1000 adrenalin chloride they reported a rapid marked increase in the number of red cells, leukocytes, and platelets in the peripheral blood associated with a diminution in the size of the spleen. Until adequate observations are available on normal subjects and individuals with other diseases, the value of the test must be regarded as problematical.

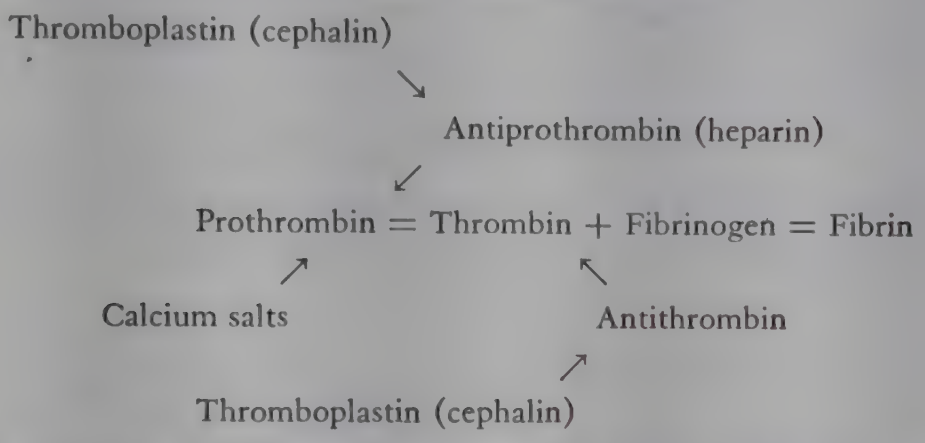
The evidence that these syndromes are caused by a disturbance of function of the spleen ("hypersplenism") is strong. Similar depressing action on the cellular constituents of the blood has been described in cases of splenomegaly due to other diseases, e.g., Gaucher's disease. Other observers, however, believe the spleen acts primarily by inhibit-

ing the formation of cells by the bone marrow rather than merely by removing them from the blood (see p. 468, idiopathic thrombocytopenic purpura).

Hemorrhagic Diseases

By hemorrhagic disease is meant any condition in which there is a tendency to abnormal or protracted bleeding. Hemorrhages due to gross anatomic lesions are not included. The tendency to bleed may be due to disturbances of coagulation, or to abnormalities of the endothelium of the capillaries, or to both.

The following diagram illustrates a commonly accepted view as to the mechanism of normal coagulation *in vitro*, based largely on Howell's theory.



Normal coagulation in the body also involves the active participation of the *platelets,* which give to the thrombus its definite structure, and are essential for its proper retraction, which in turn is necessary to bring about effective closure of the vessels. They also serve as an important source of thromboplastin. A lack of platelets also appears to result in injury to the capillary endothelium, which leads to the easy production of mechanical defects in the vessel wall, through which seepage of blood takes place.

Fibrinogen is formed in the liver. Hemorrhage due to lack of fibrinogen is met with in grave liver disease (acute hepatic necrosis and poisoning with chloroform or phosphorus). It has also been reported as a rare congenital constitutional anomaly, *fibrinopenia* ("pseudohemophilia"). It is associated with a prolonged bleeding and coagulation time, and scanty clot-formation.

A deficiency of calcium as a cause of hemorrhage has not been demonstrated and is probably impossible. Death due to disturbance of other vital functions would probably occur before a level was reached sufficiently low to inhibit thrombin formation.

Thus far there is no proof of a deficiency of thromboplastin, or an excess of heparin or antithrombin as a cause of spontaneous bleeding in man. Allen et al. (1947), however, reported finding an excess of heparin as well as a thrombocytopenia in dogs with a hemorrhagic diathesis following extensive roentgen radiation. Either protamine or toluidine blue counteracted the heparin *in vitro* and arrested the appearance of petechiae and the oozing from the mucous membranes, but there was no increase in platelets or shortening of the bleeding time and bleeding from ulcerated areas continued.

Prothrombin Deficiency. When severe enough to cause spontaneous bleeding, prothrombin deficiency is characterized by a prolonged bleeding time and coagulation time, without any alteration in the platelets. Lesser degrees of deficiency can be detected only by quantitative estimations of the prothrombin time (see p. 401).

Prothrombin is synthesized by the liver, and vitamin K is essential for this process. Bleeding due to lack of prothrombin may occur in severe liver injury because of inability to produce this substance. It may also occur as the result of a lack of vitamin (hemorrhagic disease of the newborn) or inability to absorb it from the intestine,

either because of the absence of bile salts (complete *obstructive jaundice*, bile fistulae) or disease of the intestinal mucosa (sprue, etc.). Vitamin K is synthesized by bacteria in the intestine, and except in the newborn, bleeding due simply to a deficiency of the vitamin in the diet is rare if it ever occurs. The bleeding can be checked promptly by the administration by mouth of vitamin K concentrates in conjunction with bile salts, or by parenteral injections of menadione. If the liver is severely injured, however, these measures are ineffective.

In any condition in which a reduction in prothrombin may occur, quantitative estimations should be made early, and treatment instituted before the prothrombin has fallen to a bleeding level (about 25 per cent).

Hemorrhagic Disease of the Newborn. The affection occurs in infants during the the first week of life (second to fourth day). It is not hereditary and the sexes are equally involved. The bleeding most often occurs as: (1) cutaneous hemorrhages, either oozing from abrasions or large hematomas; (2) bleeding from the umbilicus; (3) bleeding from the intestine, causing black tarry stools (both rare in hemophilia); and (4) cerebral hemorrhages which are often fatal or permanently crippling. Bleeding is often exsanguinating, and the mortality in untreated cases is from 60 to 80 per cent. Transfusion also will stop the bleeding. Recovery is complete and permanent. The bleeding can usually be prevented by the administration of vitamin K to the mother shortly before labor or to the infant on the first day.

Thrombocytopenic Purpura (Purpura Haemorrhagica). A deficiency in blood platelets is the cause of bleeding in most other cases in which the latter is due to a disturbance of coagulation. This syndrome is known as thrombocytopenic purpura. It is characterized clinically: (1) by spontaneous extravasations of blood into the skin and the mucous and serous membranes (purpura); and (2) by hemorrhages from the mucous membranes. The blood shows a normal coagulation time but a protracted bleeding time, defective clot-retraction, and a positive reaction to the capillary-resistance test. As a rule these changes are roughly parallel with the degree of platelet reduction. Bleeding usually appears if the platelet count falls below 50,000. There are exceptions, however, since bleeding may be observed with platelets at higher levels, and it may be absent or delayed with much lower counts. The bleeding is due to abnormalities in the endothelium of the small vessels which are associated with and probably caused by the reduction in platelets.

Thrombocytopenic purpura occurs: (1) as a symptom of a variety of diseases in which the activity of the marrow is depressed, the symptomatic thrombocytopenic purpuras (listed on p. 430); and (2) in the specific disease idiopathic (primary) thrombocytopenic purpura.

Idiopathic Thrombocytopenic Purpura. This disease is probably dependent upon a constitutional abnormality of the bone marrow and is sometimes hereditary. Abnormalities in the megakaryocytes, particularly a loss of granules, have been reported. They are usually plentiful. The disease affects both males and females. It usually appears in childhood, and runs a protracted chronic course, marked by recurring periods of profuse bleeding associated with a marked reduction in platelets, which may last from a few days to several weeks. These alternate with periods of remission of varying completeness and duration. As a rule symptoms subside, and the platelets rise above the critical level, although they often remain well below normal. More rarely the disease begins in an adult with an acute severe attack which may be quickly fatal. Complete permanent recovery may occur, or the usual chronic, relapsing type of the disease may follow. Bleeding

occurs most often from the nose, gums, uterus, stomach, intestine, or urinary tract. A cerebral hemorrhage may occur, but bleeding into a joint is rare. Purpuric eruptions may be absent. For a time bleeding may be restricted to a single source, as from the uterus. There is no fever or other evidence of infection. The spleen is rarely enlarged. In marrow obtained by biopsy Dameshek and Miller (1946) found the number of megakaryocytes increased, but only from 8 to 19 per cent showed evidence of platelet production as compared with about 68 per cent in normal marrow. After splenectomy, however, from 69 to 85 per cent were actively producing platelets in large numbers. They regard this as evidence that the principal cause of the thrombocytopenia is an inhibition of the production of platelets by the spleen, possibly through some "hormonal mechanism," rather than an accelerated destruction of platelets in the circulating blood.

The blood at first shows no abnormality of the red cells or leukocytes, although there may be a moderate leukopenia. An ordinary acute posthemorrhagic anemia soon develops, and frequently a secondary leukocytosis. The individual platelets are often abnormal; there are some huge platelets, while many are minute. Rarely there may be a marked neutropenia and a lymphocytosis (Minot).

TREATMENT. *Splenectomy* is usually followed by an abrupt cessation of bleeding and a critical rise in platelets to normal or above, which may be marked within a few minutes after the spleen has been removed. This fact suggests that the disease may be due to a pernicious overactivity of the spleen, manifested either by an excessive destruction of platelets in the circulation, or an inhibition of their production. Although the platelet count usually falls again after a few months, as a rule bleeding does not recur. Some cases, however, have relapsed (accessory spleens?). The operative mortality of the acute cases has been high in some clinics. Splenectomy is contraindicated in other types of purpura. Transfusions also stop bleeding, but only for three or four days. Other measures are very uncertain in their effect.

PURPURIC ERUPTIONS DUE TO ABNORMALITIES IN SMALL VESSELS. Purpuric eruptions due to abnormalities in the small vessels occur in a variety of diseases in which the platelets are normal in number, and bleeding is slight or absent. These include:

1. Intoxications with various drugs, many of which may cause a thrombocytopenia (in other patients). Many drug "idiosyncrasies" are included in this group.

2. Acute infections, such as smallpox, typhus fever, epidemic meningitis, sepsis, bacterial endocarditis.

3. Senile and cachectic purpuras.

4. Scurvy (avitaminosis). Here the bleeding is chiefly under the periosteum or into the deep muscles of the legs. There are small purpuric spots about the hair follicles of the legs. The gums and mucous membranes do not bleed.

5. *"Anaphylactoid purpura,"* which occurs chiefly in children and young adults, includes the following symptom complexes, which may occur singly, or in various combinations. Many of the patients have recurring attacks.

(*a*) Cutaneous eruptions: *erythaema multiforme,* with urticaria and angioneurotic edema, as well as purpura, or purpura alone may occur.

(*b*) *Schonlein's purpura,* a mild arthritis, most often affecting the knees and ankles, and usually showing clusters of purpuric spots about the affected joints.

(*c*) *Henoch's purpura,* characterized by recurring, acute, often severe attacks of colicky abdominal pain with tenderness and rigidity, vomiting and diarrhea or constipation, and often with some blood in the stools and vomitus. Bleeding is never profuse. Cutaneous eruptions are usually present. The attacks are accompanied by fever, slight leukocytosis, and symptoms of an acute infection. Hematuria or acute nephritis may follow. The condition has been mistaken for acute appendicitis, and operations needlessly performed. Localized edema of the wall of the intestine may occur, however, and rarely may result in intussusception or even perforation. There is no disturbance of coagulation, and no anemia. The capillary resistance test is variable, but its reaction is usually negative.

The disease resembles serum disease more than it does thrombocytopenic purpura. Differentiation from the latter is important, because transfusion and splenectomy are contraindicated.

VENOM TEST. Peck et al. (1936) reported that intracutaneous injections of moccasin venom caused local reactions in cases of thrombocytopenic purpura and certain drug purpuras during the period of active bleeding, with a disappearance of the reaction in patients who improved clinically. This was not necessarily associated with an increase in platelets or a reduction of the bleeding time. They regarded the test as of prognostic as well as diagnostic value. Recent work indicates that the test is probably significant only in indicating abnormal fragility of the small vessels. Therapeutic use of venom has given contradictory and usually disappointing results. It is probably useless in thrombocytopenic purpura.

PROCEDURE. One-tenth ml. of a 1 : 3000 dilution of standard venom (obtainable from Lederle Laboratories) is injected intracutaneously. A positive reaction is indicated by the appearance of an ecchymosis at least 1 cm. in diameter at the site of the injection within an hour. An injection of salt solution is given as a control.

Hemophilia. This is a rare, hereditary disease, due, possibly, to a constitutional abnormality of the platelets. It is characterized by a tendency to protracted bleeding from slight cuts and bruises. The bleeding often persists for days or even weeks as a slow oozing which may eventually be exsanguinating. Bleeding often occurs also into the subcutaneous tissues and into the joints, which are seriously damaged after repeated attacks, but rarely from the mucous membranes, except from the nose or gums. Many victims have died from hemorrhage following circumcision or tooth extraction. The tendency to bleed is always manifested in infancy (although rarely during the first week), and patients with the severer manifestations usually die during the first year. The tendency to bleed varies markedly at different times in the same individual.

Bleeding does not occur from a simple pin-prick, and venepuncture can be performed without danger. The bleeding time is usually normal, and reaction to the capillary resistance test is negative. Purpuric eruptions never occur. The coagulation time usually is greatly prolonged, often to an hour or more. However, this varies greatly in the same individual on successive examinations, and at times may be nearly normal. The clot retracts normally after it once forms. The platelets are normal in number, and there is no reduction in the quantity of fibrinogen, calcium, or prothrombin. There is a delay in the conversion of prothrombin into thrombin. There is a deficiency in hemophilic plasma of some clot-promoting substance (thromboplastin, Howell) which is present in normal plasma and is largely concentrated in Cohn's fraction I, and subfraction II of fraction III of the plasma globulins. It is associated with but separable from fibrinogen and prothrombin. Many have believed that the platelets are abnormally resistant and do not disintegrate and initiate coagulation by liberation of thromboplastin, as do normal platelets. Recent work, however, casts doubt on this view. Cytologically the blood is normal or shows the changes of an ordinary posthemorrhagic anemia.

The bleeding may be checked for three or four days by transfusion, or by injections of the antihemophilic globulin fraction of normal plasma.

The disease occurs only in males, and direct transmission is solely through females, the conductors, as a recessive, sex-linked character. The sons of a hemophilic male never have the disease, and never transmit it, but his daughters become conductors. Of the children of a conductor, half her sons manifest the disease, and half her daughters become conductors, and transmit the defect. Theoretically half the children, male and female, of a hemophilic male and a conductor should have the disease, but thus far no authentic cases in females have been observed.

Hereditary Pseudohemophilia (von Willebrand's syndrome, 1929). This is a rare, usually familial disease affecting both males and females and transmitted by both sexes, probably as a simple incomplete dominant character. It is characterized by profuse

bleeding from the nose, gums, cuts, bruises, operative incisions, and after dental extractions; by ecchymoses and sugillations but not by petechiae; by menorrhagia and postpartum hemorrhage; and less often by bleeding from the stomach, intestine, and urinary tract. Hemarthrosis is rare. Bleeding may be present at birth and usually appears during early childhood. It is not infrequently fatal, but if the individual survives to adult age, the bleeding tendency often lessens. It is somewhat more common in females, and the fatal cases reported have been almost exclusively in females. Marked fluctuations in the severity of the bleeding occur.

The bleeding time is prolonged in varying degree, but clot retraction is usually normal. The tourniquet test is variable, but usually positive in severer cases. Platelets are normal or increased in number and probably are normal in function. The coagulation time and prothrombin concentration are normal. The spleen is not enlarged, and splenectomy, tried in a few cases, is ineffective and dangerous. Superficial bleeding usually can be controlled by local applications of fibrin foam and thrombin solution, but transfusions have little if any effect in checking bleeding. Severe bleeding, persisting in spite of transfusions, often follows operations of any type, and the latter should be avoided unless imperatively necessary.

The mechanism of the bleeding has not been established. Macfarlane (1941) and others have described abnormalities in the minute vessels of the nail beds. The capillary loops are distended and tortuous, and if a single loop is punctured, it fails to constrict in the normal manner and permits continued oozing of blood into the tissues. The possibility of some coagulation defect has not been excluded, but none has been satisfactorily demonstrated.

Hereditary Hemorrhagic Thrombasthenia. Under this title Glanzmann (1918) described a type of familial hemorrhagic disease which clinically resembled hereditary pseudohemophilia. The coagulation factors (including the bleeding time) were all normal except for defective clot retraction, which he attributed to a qualitative defect in the platelets. His observations have not been confirmed.

Many individual cases of hemorrhagic disease have been reported, which do not correspond exactly to any of the syndromes described, and which cannot be satisfactorily classified. Some have questioned the possibility of a precise classification, since notable differences may be observed between two bleeders in the same family and even in the same individual on different occasions.

PART III

Parasitology

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Chapters 16–29

CHAPTER 16

Medical Parasitology

Parasitology is the science which deals with parasites, their activities and effects. A parasite is an organism that lives on or within and at the expense of some other living organism. We are concerned here with the animal parasites of man, or *medical parasitology*. All the parasites of man belong to one of the following major animal groups, and the science of parasitology is often divided accordingly (see p. 476).

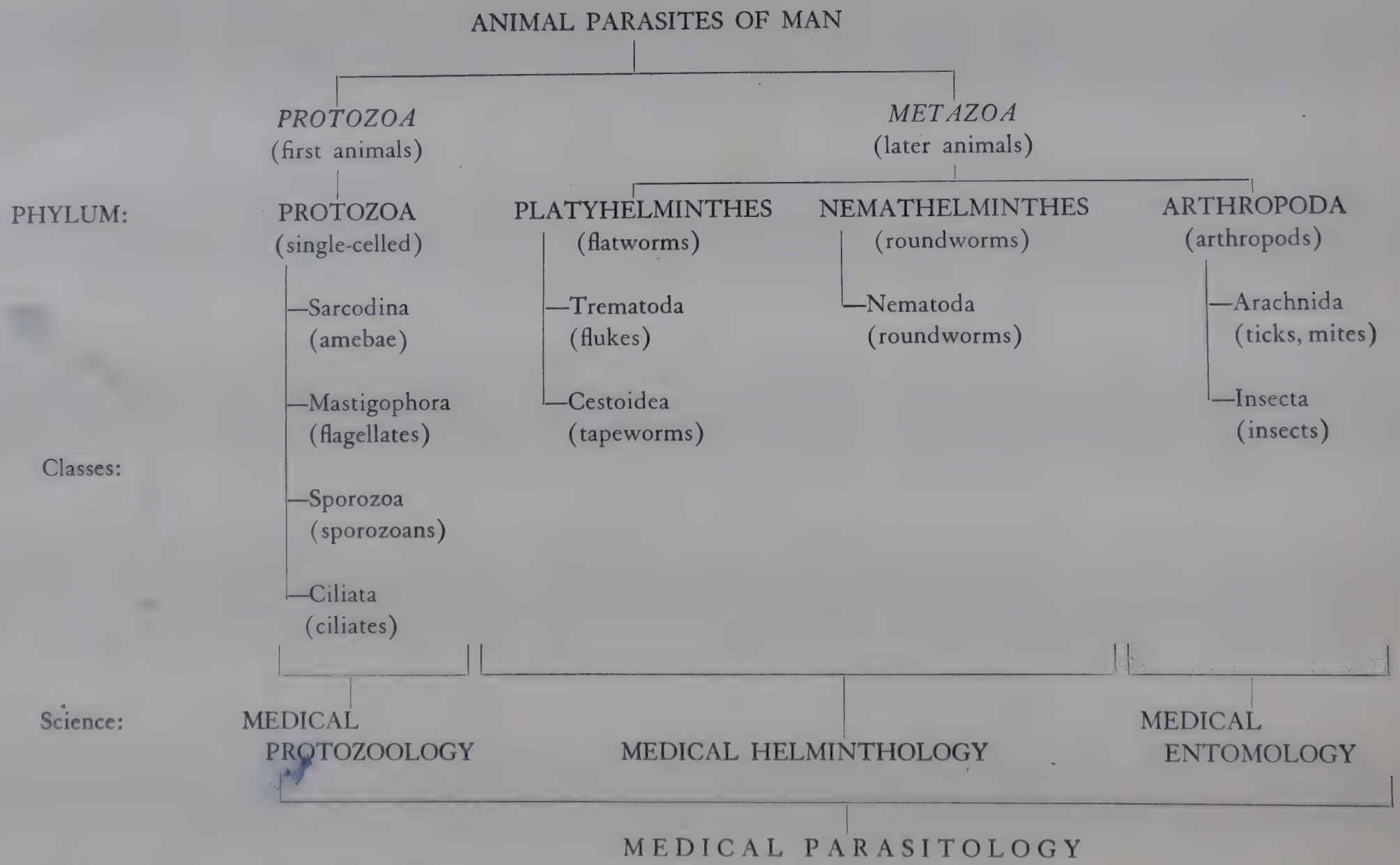
Terminology. There are certain terms employed in parasitology which it is necessary to understand. Among these, the following are explained:

The animal which supports the parasite is known as the *host*. Many parasites utilize different hosts for different stages of development. The animal harboring the adult stage is referred to as the *definitive* host, that harboring the larval stage as the *intermediate* host. *First* and *second*, or more, intermediate hosts may be required when there is more than one larval stage. Some parasites utilize several different hosts during their life, each host serving the parasite in the same manner. Thus we speak of three-host ticks although the parasite-host relationship is not changed during the parasite's life cycle: each host provides the parasite with a blood meal.

Some parasites depend entirely upon their host or hosts for continued existence; these are called *obligatory* parasites. They may survive outside the host for varying periods of time in a resistant stage, but they must return to a host in order to continue life and reproduction. Other parasites are capable of either independent existence or parasitic life; these are referred to as *facultative* parasites.

The term *true parasitism* implies a condition in which the parasite does harm to the host and derives all benefit from the association; an example of this would be the hookworm infecting man. *Commensalism* is a term used when the parasite is benefited and the host unharmed; the nonpathogenic protozoa of the human intestinal tract provide examples.

At times we divide parasites into *ectoparasites* and *endoparasites* according to their habitat upon or within the body of the host. It would be an easy matter to decide that the body louse belonged to the first group and the hookworm to the latter; but it is more difficult with the itch mite which penetrates the skin. It is usual to apply the term *infection* for parasitization within the body and *infestation* for that which is external or visible. Using the illustrations above, an individual is infected by hookworm and infested by lice; parasitization by the itch mite is usually called an infection.



The thorny-headed worms (Acanthocephala), the hairworms (class Gordiacea of the phylum Nematomorpha), and the leeches (class Hirudinea of the phylum Annelida) are occasionally parasitic and will be briefly discussed.

Parasites of other animals or plants may enter the body of man; when man plays no part in the life cycles of such organisms they are called *accidental* parasites.

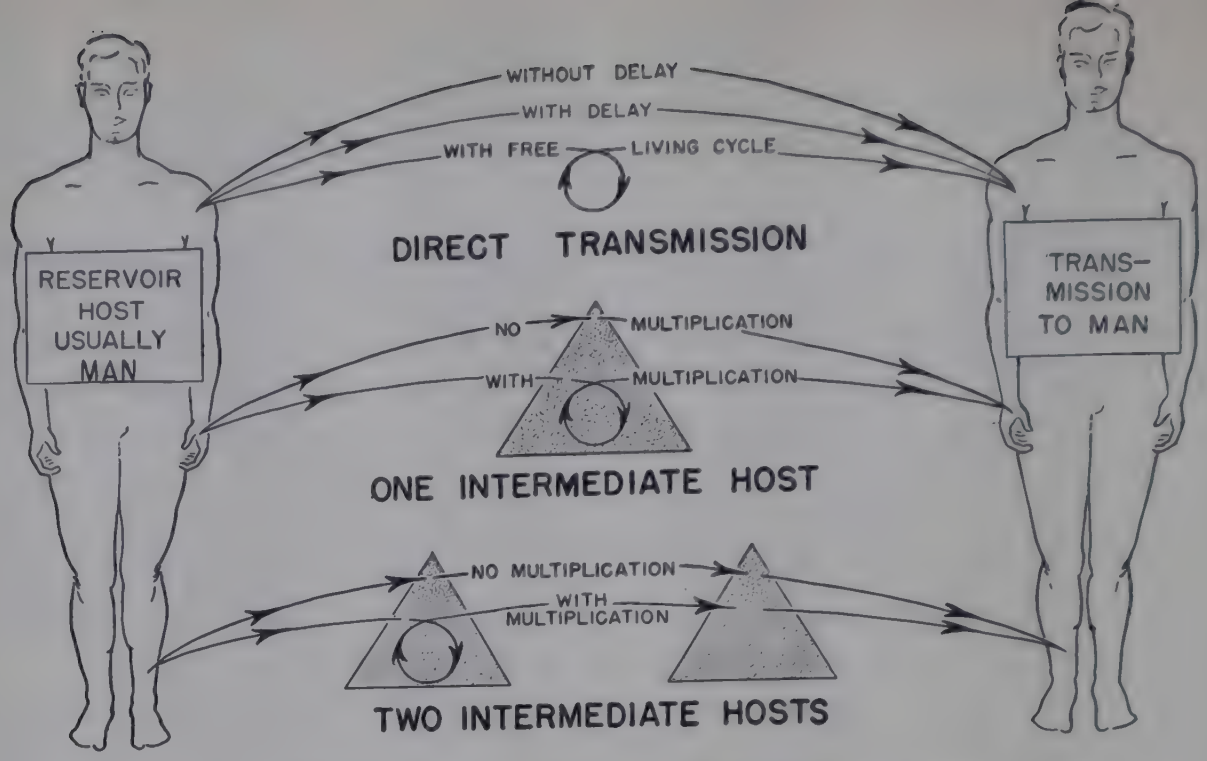
Epidemiology and Life Histories. The life histories of parasites which show direct transmission as from man to man, or reinfection of the same individual, are on the whole simpler than those requiring one or more intermediate hosts; yet there is great variety in the complexity of the life cycles of the former. The eggs of the pinworm are infective for the host soon after they are deposited on the perianal skin; eggs of the common roundworm must incubate in a favorable environment until an embryo has developed within the shell, when they prove infective upon ingestion; with the hookworm egg, not only must an embryo develop within the egg shell, but this embryo must also leave the shell, undergo molting, and change in form before it is capable of producing a new infection.

With the parasites requiring one or more intermediate hosts the problem is still more complex. The cycles may vary from the relatively simple case of the beef tapeworm, in which the embryo develops in the muscles of the cow and the adult in man, to the involved cycle of the human lung fluke in which the larval stages are passed in the snail and then in the crab.

It will be evident that, should the entire life cycle of an animal parasite be passed in a single host, the continuation of the species would be endangered—the parasite would die when the host ceased to live. For this reason the transfer of parasites to another individual is necessary, or there must be a substitution of hosts or an adoption of free-living periods. Where parasites must pass from one host to another and back to the original host the chances of individual survival are very slight. To provide against extinction of species there is prodigality in reproduction. Some parasites show reproduction only by the adults in the definitive hosts; others show multiplication in both definitive and intermediate hosts. The more common roundworms of man produce a large number of eggs without multiplication in the intermediate stages; the flukes produce large numbers of eggs in the definitive host, followed by extensive multiplication in the snail; the malaria parasites multiply in both man and mosquito.

The parasite frequently assumes a resistant stage as it leaves the definitive host. This is especially true of those that have but a single host, or that have vertebrates for both definitive and intermediate hosts. The resistant stage is passed from one host to another when it is *ingested*. The passage of parasites from vertebrate to arthropod is also ingestive, but the passage back to the vertebrate is usually by *inoculation;* it may be by ingestion when a fly deposits the infective stage on man's food.

Various arthropods thus become *vectors* of parasitic diseases. The term *mechanical vector* is applied to those in which the parasite is unchanged in its transfer from host to host. Infection may be introduced into the new host through the vector's feces or by its contaminated appendages; the common housefly carrying protozoan cysts is an example. Many of the parasitic diseases are transmitted by *biologic vectors,* of which we have two types: (1) those in which the parasite



Direct Transmission
(Intermediate host not required)

Without delay—
parasite immediately infective for man:
- *Endamoeba histolytica*
- *Giardia lamblia*
- *Trichomonas vaginalis*
- *Balantidium coli*
- *Plasmodium* species (under special circumstances)*
- *Trypanosoma* species (under special circumstances)
- *Leishmania* species (under special circumstances)
- *Hymenolepis nana*
- *Sarcoptes scabiei*
- *Pediculus humanus*
- *Phthirus pubis*

With delay—
parasite must develop to infective stage:
- *Enterobius vermicularis*
- *Ascaris lumbricoides*
- *Trichuris trichiura*
- *Ancylostoma* species
- *Necator americanus*
- *Strongyloides stercoralis*
- *Trichinella spiralis*

With free-living cycle—
parasite must develop to infective stage:
- *Strongyloides stercoralis*

One Intermediate Host
(Obligatory)

No multiplication but parasite undergoes development in intermediate host:
- *Wuchereria* species
- *Onchocerca volvulus*
- *Loa loa*
- *Dracunculus medinensis*
- *Taenia* species
- *Dipylidium caninum*
- *Hymenolepis diminuta*
- *Hymenolepis nana* (rarely)

With multiplication and development in intermediate host:
- *Schistosoma* species
- *Trypanosoma* species
- *Leishmania* species
- *Plasmodium* species*
- *Echinococcus granulosus**

Two Intermediate Hosts
(Obligatory)

No multiplication but parasite undergoes development in both intermediate hosts:
- *Diphyllobothrium latum*

With multiplication in first intermediate host—development in both:
- *Clonorchis sinensis* and other liver flukes
- *Fasciolopsis buski* and other intestinal flukes
- *Paragonimus westermani*

*In these species man is the intermediate host and some other animal, the definitive host. The life cycles, however, fall easily into the above scheme.

undergoes biologic changes without increasing in numbers (the mosquito transmitting filariasis); (2) those in which the parasite shows multiplication as well as biologic changes (the mosquito carrying malaria).

The source from which new hosts are parasitized is referred to as the *reservoir host*. Man is the only reservoir host in malaria, mosquitoes becoming parasitized when they ingest human blood containing sexual forms of the parasite. In certain other diseases, some other animal as well as man may serve as the reservoir of infection; in a few, lower animals are the principal reservoir, man being only secondarily involved. In searching for the reservoir hosts one should keep in mind that the parasites of man often have closely related species in other animals.

The epidemiology of parasitic diseases is seen to present a complex problem involving the life history of each parasite and the various methods by which the infective stages reach the human host. Racial or individual habits, environment, and the degree of civilization all have their part in determining the distribution of each parasite. Any life history involving other animals as hosts or vectors will influence the incidence in man accordingly. Because of the complexity of the problem, the life history of each important parasite will be discussed hereafter in detail.

Classification. All living things are divided into groups according to similar morphologic characters. The largest divisions are the *kingdoms,* and most living things fall into either the animal kingdom or the plant kingdom; some—viruses and rickettsiae—do not fit well into either. Each kingdom is divided into branches or *phyla* and subdivided into *classes, orders, families, tribes, genera,* and *species.* Although occasionally divided into *subspecies,* the species is the basic unit of classification.

A *species* is defined as a group of similar individuals which can mate and produce fertile young. In some instances the female of one species may mate with the male of a related species, but the offspring, a hybrid, is sterile. Of course, the male and female of the same species may be very unlike, but the young resulting from mating will have characters similar to the parents. The male is designated by the sign of Mars, ♂, and the female by the sign of Venus, ♀.

Species which are very much alike are grouped into a *genus,* and the species representative of that genus is called the *type species;* the definition is based on the first-described species of that genus; if at any time such a genus is broken up into other genera, this *genotype* remains with the original. When a valid name is given to a species and that species is later transferred to another genus, the specific cognomen must attach to the new genus. Thus *Culex aegypti,* when placed with the genus *Stegomyia,* became *Stegomyia aegypti* and, when placed with the genus *Aëdes,* became *Aëdes aegypti.* A genus may have only a single species.

The term *tribe* is applied to certain genera that show resemblances permitting broad groupings; the name of a tribe ends in "ini." Tribes with common characters may be grouped into *subfamilies* with a name ending in "inae." The latter are similarly grouped into *families,* the name ending in "idae." Many families are not divided into subfamilies, being separated directly into genera; at times they are grouped into a *superfamily,* the termination being "oidea."

An *order* is formed by a group of families agreeing in some striking feature, and a *class* is similarly formed from orders. The following are examples of classification:

	Man	*A Mosquito*	*A Malaria Parasite*
KINGDOM:	Animal	Animal	Animal
PHYLUM:	Chordata	Arthropoda	Protozoa
CLASS:	Mammalia	Insecta	Sporozoa
ORDER:	Primates	Diptera	Haemosporidia
FAMILY:	Hominidae	Culicidae	Plasmodiidae
SUBFAMILY:		Culicinae	
TRIBE:		Anophelini	
GENUS:	*Homo*	*Anopheles*	*Plasmodium*
SPECIES:	*sapiens*	*punctulatus*	*vivax*

Nomenclature. When the thousands of different genera and species of animals are considered, it will be readily perceived that indescribable confusion would prevail unless some system existed for their designation. To avoid this, the International Code based on the rules of Linnaeus (tenth edition of Systema Naturae, 1758) is made the basis of binary zoologic nomenclature. Both generic and specific names are used in referring to all living things. In naming a species, the genus which has a Greek or Latin name commencing with a capital is written first and is followed by the specific name which begins with a small letter. Accordingly, the scientific name of man is *Homo sapiens*. In this case the genus is monotypic, there being only one species.

For the generic name to be valid, it must be one that has not already been given to another group of animals. It is a rule of nomenclature that zoologic names are independent of botanic ones so that prior use of a generic name for a plant is not a valid objection to its use for an animal; but it is well to avoid introduction into zoology of generic names which are already in use in botany.

The specific name may be a noun in the genitive; if an adjective, it must agree in gender with the generic name. It is permissible to have a masculine noun as the specific name with a feminine generic name. If the specific name is a modern patronymic, we add "i" in the case of a man or "ae" for a woman to the exact and complete name of the person.

In printed matter the zoologic name should be in italics, that of the family in Roman type. Names should be Latin or latinized. The name of the author of a specific name is written immediately after the scientific name, without punctuation, and may be followed by the year of publication set off by a comma, thus: *Ascaris lumbricoides* Linnaeus, 1758. Should the name of the author appear in parentheses, this indicates that he proposed the specific name but placed the species in a genus other than that in which it now appears; the name of the author responsible for placing the species in the present genus may be written after the name of the original author of the species. For example, "*Schistosoma haematobium* (Bilharz, 1852) Weinland, 1858" tells us that Bilharz proposed the specific name *haematobium* in 1852 but placed it in some other genus and that Weinland in 1858 transferred it to the genus *Schistosoma.*

There are certain rules governing the naming of animals. Of these, the law of priority provides that the oldest published name, under the Code, of any genus or species is its proper zoologic name. The name must appear in a well-recognized publication, and even the printer's proof does not establish priority. In the case of larva and adult, or male and female, formerly considered different animals but subsequently found to be the same, the oldest available name becomes the name of the species. Another point is that names are not definitions; consequently the lack of appropriateness of any name is no objection to its continued use. This will appeal to anyone as a wise provision, for if a different name

were substituted each time a designation more descriptive or applicable was invented, it would be utterly destructive to system. Considering that some of our parasitic animals have approximately 50 different designations, it becomes important to eliminate all but the single, proper zoologic name.

The objection so frequently heard in connection with adopting new names for old ones is not well founded. Wherever confusion has reigned, the establishment of order always results in temporary greater confusion. The student taking up this subject a few years hence will have to burden his mind with only one name for each parasite. There is only one correct name for an animal and all others are synonyms. The principal cause of changes in names is that our conception of the relationships of animals changes.

Keys. A biologic key is a series of alternative or contrasting statements forming a set of directions which, when correctly followed, lead to the identification of the unclassified organism. Keys are usually constructed in an abbreviated or outline form and may assume various mechanical structures, depending upon their length and complexity. The simplest key, and the one used in this section, consists of a series of couplets of contrasting possibilities, only one of which will be true for the animal to be identified. In using a key, one must choose the correct alternative and follow where it leads. The key below is for the larger groups of the animal parasites of man. Keys to the important genera and species will be found in appropriate chapters.

Key to Animal Parasites of Man
(Modified from Stiles)

1. Unicellular animals (acellular); without tissues; *protozoa* 2
   Multicellular animals; with tissues; *metazoa* 5
2. Animals with locomotor organelles 3
   Animals without locomotor organelles; both sexual and asexual reproduction, the former resulting in sporozoites; *sporozoans* ..... Class Sporozoa
3. Animals moving by means of filamentous or hairlike organelles 4
   Animals moving by means of rootlike extensions of cytoplasm or pseudopodia; *amebae* ..... Class Sarcodina
4. Animals moving by means of threadlike or whiplike filaments, flagella; *flagellates* ..... Class Mastigophora
   Animals moving by means of fine, short filaments—cilia—covering the entire body; *ciliates* ..... Class Ciliata
5. Body never provided with jointed appendages 6
   Body with jointed appendages in the adult; provided with mouth parts; breathe through tracheae 11
6. Body more or less flattened 7
   Body ordinarily round in cross section 9
7. With complete alimentary canal—mouth, intestine, and anus present; sucker on posterior end; body annulated like an earthworm; parasitic in upper respiratory tract or externally; *leeches, bloodsuckers* ..... Class Hirudinea
   Without complete alimentary canal—mouth and intestine present or absent, always without anus 8

8. Body of adult segmented; two or four suckers present; alimentary tract absent; tissue usually contains calcareous corpuscles; adults parasitic in intestines; *tapeworms* ..... Class Cestoidea
   Body of adult not segmented; one or two suckers present; mouth and intestine present; parasitic in liver, lungs, blood, intestine, occasionally elsewhere; *flukes* ..... Class Trematoda
9. Intestine present; no armed rostellum 10
   Intestine absent; armed rostellum present; very rare in man, in intestine; *thorny-headed worms* ..... Acanthocephala
10. Intestine rudimentary in adult; lateral chords absent; rare, accidental parasite in intestine of man; *hairworms* ..... Class Gordiacea
    Intestine complete in adult; lateral chords present; parasitic in many organs and tissues; very common and important; *roundworms* ..... Class Nematoda
11. Body of adult divided into distinct regions: head, thorax, and abdomen; six legs present in adult; larva annulated much like an earthworm; *insects* ..... Class Insecta
    Body not divided into distinct regions; head, thorax, and abdomen coalesced; eight legs in adult, six in larva; larva not annulated; *ticks* and *mites* ..... Class Arachnida

CHAPTER 17

Medical Protozoology

Protozoa Causing Specific Human Diseases

Table 51

PROTOZOA CAUSING SPECIFIC HUMAN DISEASES

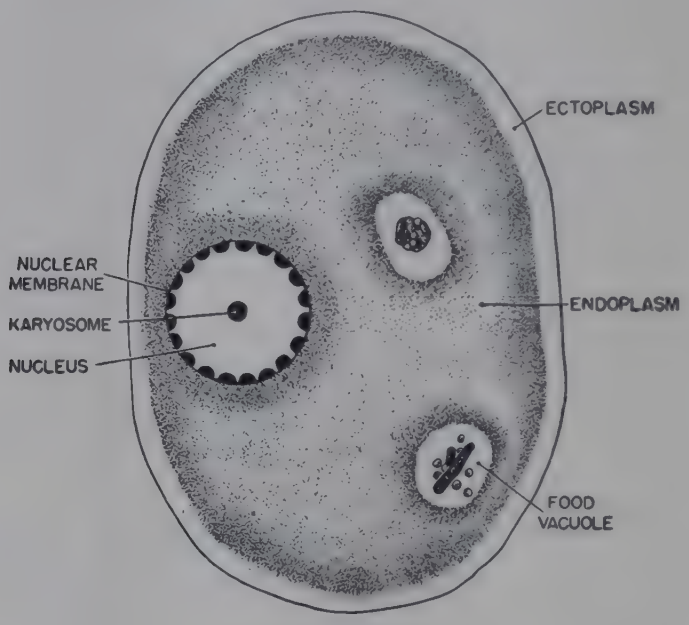
Parasites (Diseases)	*Definitive Hosts*	*Intermediate Hosts*	*Important Reservoir Hosts*	*Transmission to Man*
Endamoeba histolytica (Amebiasis)	Man	None	Man	By ingestion (mature cyst)
Balantidium coli (Balantidiasis)	Hog, man	None	Hog, man	By ingestion (mature cyst)
Giardia lamblia (Giardiasis)	Man	None	Man	By ingestion (mature cyst)
Trichomonas vaginalis (Trichomonad vaginitis)	Man	None	Man	Not known
Trypanosoma gambiense *Trypanosoma rhodesiense* (African sleeping sickness)	Man, animals	Tsetse flies (*Glossina* sp.)	Man, animals	By inoculation (bite of fly)
Trypanosoma cruzi (Chagas' Disease)	Animals, man	Reduviid bugs	Armadillo, opossum	Infective feces (deposited by bug)
Leishmania donovani (Kala-azar) *Leishmania tropica* (Oriental sore) *Leishmania brasiliensis** (Espundia)	Man, dog	Sandflies (*Phlebotomus* sp.)	Dog, man	By inoculation (bite of fly; direct transmission possible)
Plasmodium malariae *Plasmodium vivax* *Plasmodium falciparum* *Plasmodium ovale* *Plasmodium knowlesi* (Malaria)	Anopheline mosquitoes	Man	Man	By inoculation (bite of mosquito; also by transfer of infected blood)

*While dogs and other animals have been implicated in *Leishmania donovani* and *L. tropica*, no animal host has been proved for *L. brasiliensis;* this also applies to the insect host.

Morphology and Biology. By the term "protozoa" we understand a group of animals in which the individual is composed of a single cell morphologically and functionally complete; it is not one of a number of cells going to make up a complex individual and dependent upon such a combination as is the case with

metazoa. There is no differentiation into tissues, the single cell serving all functions necessary for existence.

Cytoplasm. The cytoplasm may be separated into an external hyaline portion, the ectoplasm, and an inner granular portion, the endoplasm. The ectoplasm serves as a protective covering and provides skeletal and supporting structures; it may be modified into *organelles* that are concerned with locomotion and food capture. We recognize three means of locomotion—pseudopodia, flagella, and cilia—and these are used in separating the Protozoa into classes. The endoplasm contains various inclusions—nuclei, food vacuoles, contractile vacuoles, and other structures concerned with reproduction and nutrition.



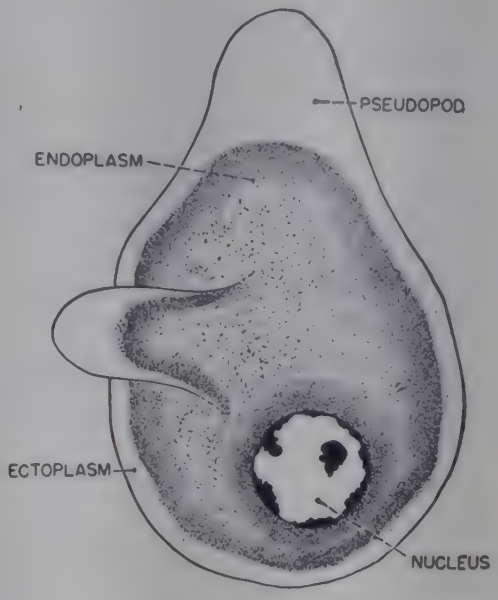
A protozoan.

Nucleus. Most protozoa contain a single nucleus although each of the four classes contains species provided with more than one. The nucleus is concerned chiefly with reproduction and is characterized by concentration of the so-called *chromatin substance* of the cell. The usually accepted test for chromatin, the staining affinity for basic aniline dyes, is not entirely satisfactory as substances other than chromatin may stain even more intensively; such material is usually extranuclear and is identified as *chromatoid* (chromatin-like).

The nucleus is made up of a network of *linin,* an achromic reticulum in which the nuclear sap or karyolymph is contained. An achromic nuclear membrane usually separates the nucleus from the cytoplasm. In addition there is an achromic substance, plastin, in which the chromatin grains are embedded. *Karyosomes* are made up of plastin-chromatin combinations; they are dark-staining and usually located at or near the center of the nucleus. Chromatin grains may be scattered throughout the nuclear area, on the nuclear membrane, or on the network of linin; when scattered throughout the cytoplasm as extranuclear granulations such granulations are called chromidia.

A *centrosome* which presides over cell division is usually located just outside the nucleus. In some protozoa, however, the centrosome is within the nucleus and

may be seen inside the karyosome, then being called a centriole. The centrosome may also function in kinetic activities and is then called a blepharoplast. When appearing as a small granule at the base of the flagellar apparatus, it is called a basal granule.

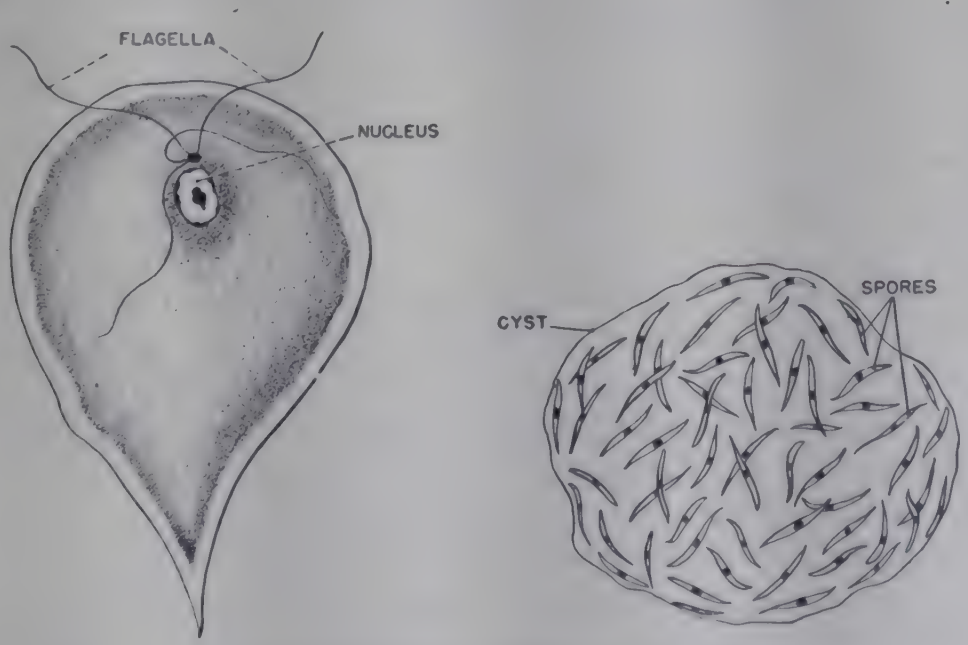


An ameba.

Reproduction. Two main types of reproduction are seen in the Protozoa. Asexual multiplication is accomplished by simple division or *fission*—when the nucleus and the cytoplasm divide into two; or the nucleus may divide into a number of daughter nuclei, this division being followed by multiple splitting of the cytoplasm, *schizogony*. Sexual reproduction follows the differentiation of certain cells into male and female forms. The union of the two sexual cells, *syngamy,* results in the formation of a new individual, the *zygote*. An additional type of sexual reproduction is found in the ciliates and consists of a temporary fusion or conjugation of two similar animals.

Classification: Sarcodina. This class contains one family of medical importance, whose members are commonly called the amebae. The various forms of this class move by means of pseudopodia—rootlike extensions of the cytoplasm—which also serve as structures for obtaining food. Ectoplasm or both ectoplasm and endoplasm may take part in making up the pseudopodia. These organelles vary in shape and characteristic activity in the different species.

Mastigophora. This class contains several families of human protozoa, all belonging to the same order. The motile forms of this class have threadlike or whiplike processes, *flagella,* for the purpose of locomotion and obtaining food. Some species have an undulating membrane in addition to the flagellum.



(*Left*) A flagellate. (*Right*) A sporozoan.

Table 52

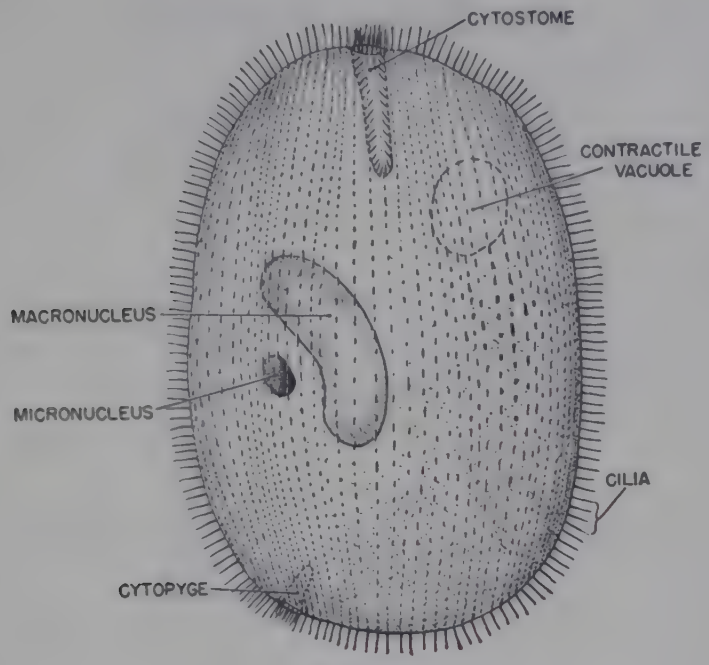
CLASSIFICATION OF THE PROTOZOA OF MAN

Class	*Order*	*Family*	*Genus and Species*
Sarcodina	Amoebida	Endamoebidae	*Endamoeba histolytica* *Endamoeba coli* *Endamoeba gingivalis* *Endolimax nana* *Iodamoeba bütschlii* *Dientamoeba fragilis*
Mastigophora	Protomonadida	Cercomonadidae	*Enteromonas hominis*
		Chilomastigidae	*Chilomastix mesnili*
		Embadomonadidae	*Retortamonas intestinalis*
		Octomitidae	*Giardia lamblia*
		Trichomonadidae	*Trichomonas hominis* *Trichomonas vaginalis* *Trichomonas tenax*
		Trypanosomidae	*Trypanosoma gambiense* *Trypanosoma rhodesiense* *Trypanosoma cruzi* *Leishmania donovani* *Leishmania tropica* *Leishmania brasiliensis*
Sporozoa	Coccidia	Eimeridae	*Isospora hominis* *Eimeria stiedae*
	Haemosporidia	Plasmodiidae	*Plasmodium malariae* *Plasmodium vivax* *Plasmodium falciparum* *Plasmodium ovale* *Plasmodium knowlesi*
Ciliata	Heterotrichida	Bursaridae	*Balantidium coli*

SPOROZOA. This class is identified by the method of reproduction and by the absence of locomotor organelles. Reproduction may be *asexual* (schizogony) or *sexual*—after union of male and female gametes and the production of spores (sporogony). All the Sporozoa of man are parasitic within cells, tissues, or body cavities. This class includes the important malaria parasites.

CILIATA. Only one species of medical importance is included in this class. These animals are characterized by a covering of hairlike cilia which serve for locomotion and to direct food particles into the mouth; the organelles may be evenly distributed over the entire animal or more prominent in certain regions. The body

is enveloped in a cuticle which may have only one opening, the cytostome (mouth), or a second opening, the cytopyge (anus). The organisms usually have both a large *macronucleus* and a small *micronucleus*.



A ciliate.

Plan of Study of Protozoa of Man. By comparing Tables 51 and 52, it is immediately apparent that not all of the protozoa infecting man cause disease. Some are true parasites while others are commensal organisms. Since the nonpathogenic forms may be confused with the specific etiologic agents of certain diseases, medical protozoology must include a study of all the protozoa to be found in man.

The protozoa of man of major importance readily fall into two groups based upon their habitat in the body—the blood protozoa and the intestinal protozoa; a third group of less importance is made up of those inhabiting other parts of the body. These groups provide a better classification for clinical and laboratory study than do their zoologic relationships. For this reason the material pertaining to medical protozoology will be presented under the headings of Blood Protozoa and Intestinal Protozoa; the other protozoa are included with related organisms of the latter group.

CHAPTER 18

The Blood Protozoa

The protozoa found in the blood of man may be divided into two groups. The malaria parasites (sporozoans) comprise the group of major importance; hemoflagellates of the class Mastigophora are found in blood and tissues and make up the second group.

The Malaria Parasites

Malaria has formerly been considered an important problem of only tropical and subtropical climates, especially those of foreign lands. With the recent exposure of military forces to this disease in many regions of the world and their return to civilian life, malaria is no longer an exotic disease. It has always been the most important of human diseases caused by Protozoa, and World War II has increased this importance; almost everyone will be personally acquainted with some person who has had or is still having attacks of malaria. Every laboratory technician must know the malaria parasite; an accurate and reliable diagnosis of the disease rests solely upon the use of the microscope by an adequately trained individual. The history of malaria in the United States is covered in an interesting paper by Faust (1945).

Classification. The order Haemosporidia includes two families and several genera, to one of which the malaria parasites belong. This genus, *Plasmodium* Marchiafava and Celli, 1885, is characterized by parasites which invade red blood cells, showing both asexual reproduction (schizogony) and gametocyte formation within them; which exhibit ameboid activity at certain stages; and which produce pigment. Species of *Plasmodium* related to those found in man have been demonstrated in other vertebrates, especially in monkeys and birds. More distantly related parasites are also found in other vertebrates. Of these may be mentioned *Babesia* in cattle, sheep, horses, and other mammals; *Haemoproteus* in birds and cold-blooded vertebrates; and *Leucocytozoon* in birds.

The following species are recognized as causing disease in man:

Plasmodium malariae (Laveran, 1881) Grassi and Feletti, 1890.
Plasmodium vivax (Grassi and Feletti, 1890).
Plasmodium falciparum (Welch, 1897).
Plasmodium ovale Stevens, 1922.
Plasmodium knowlesi Stinton and Mulligan, 1932.

The recognition of *strains* or *races* of the various species of *Plasmodium* is based on observations of clinical virulence, infectivity for man or mosquitoes, and re-

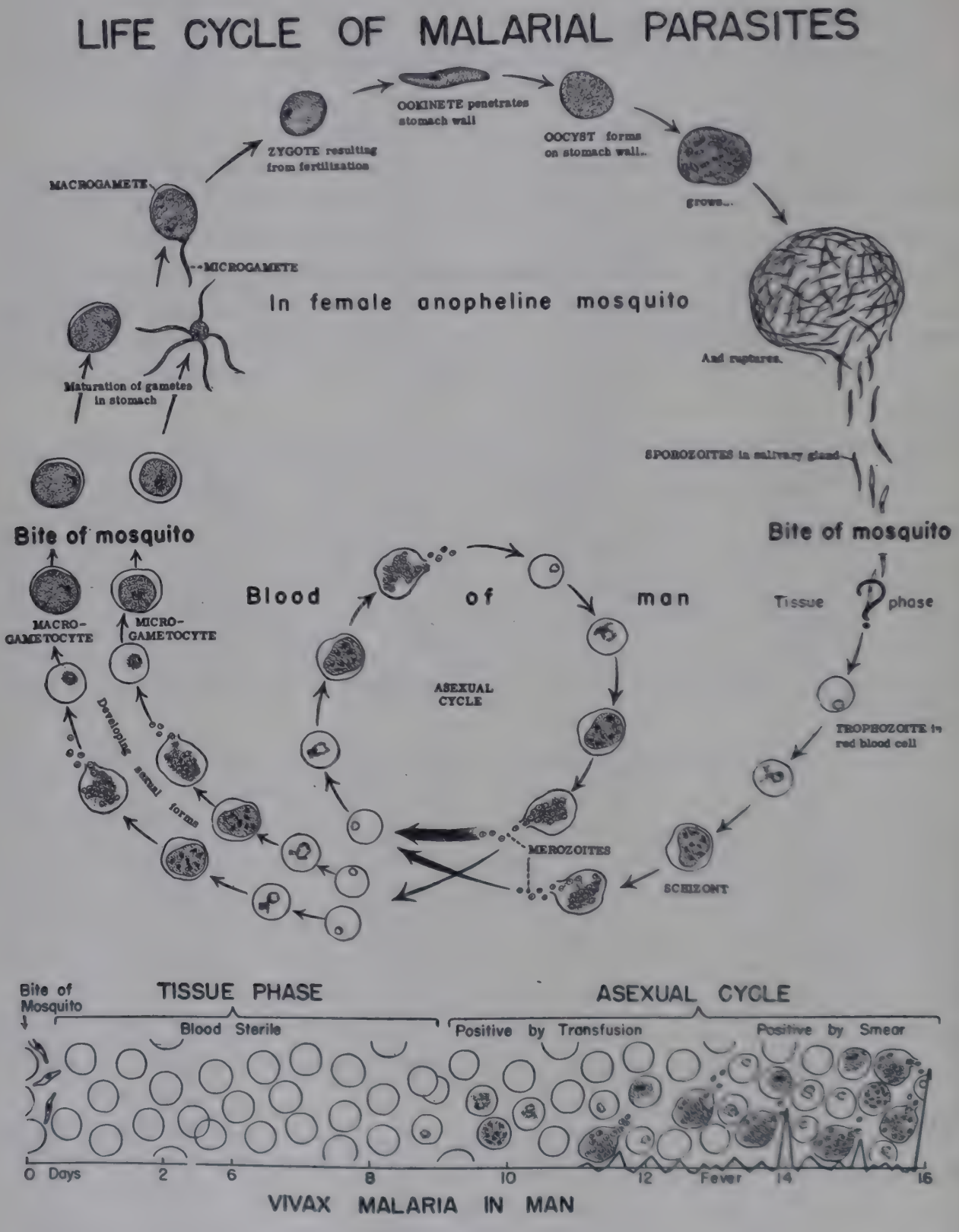
lated immunologic differences. Such features have not been correlated with morphologic characters. Coatney and Young (1941) list the following strains as studied in the United States: the McCoy, Cleveland, St. Elizabeth, and U. S. Public Health Service strains of *Plasmodium vivax;* the Jones strain of *P. malariae;* and the Long strain of *P. falciparum*. More recent studies have included strains of *P. vivax* acquired in New Guinea and the Solomon Islands.

Life History and Morphology. In general, the plasmodia parasites are host specific: human species are not infective for monkeys and species from the monkey do not develop in birds. *Plasmodium knowlesi,* normally parasitic in the macacus monkey, is an exception, being capable of producing experimental disease in man. The plasmodia are also host specific in the mosquito: only anophelines serve as vectors in human malaria, while culicine mosquitoes are the definitive hosts for species found in birds. The definitive hosts for many species are not known.

Two cycles of development are involved in human malaria: one in the definitive host, the female anopheline mosquito, and one in the intermediate host, man. These two cycles are called sexual (sporogonous) and asexual (schizogonous), respectively, and the complete life cycle requires an alternation between the two. Although the sexual cycle begins in man with the formation of gametocytes, it cannot be completed until these sexual forms have been ingested by the mosquito. After a period of development and multiplication within the insect, infective stages may be introduced into a new intermediate host when the mosquito again feeds.

Cycle in Man. The infective stage for man, the *sporozoite,* is introduced with the salivary fluid of the biting mosquito. Repeated experiments on man and birds by various authors have shown that the parasites are inoculated into tissues as well as directly into the blood stream. Immediate surgical removal of the bitten area does not prevent infection, indicating that parasites are promptly carried to distant points; tissue removed from the area of the bite is found to be infective; and mosquitoes biting through skin over a blister also produce infection. More recently, Fairley et al. (1945), working with Australian soldiers, have shown that the sporozoites can be demonstrated in the circulating blood immediately and for several minutes following the bite; after this brief period, parasites are absent for about seven days or longer.

The development of the parasite during the period from its disappearance to its reappearance in the blood stream is not known. Research conducted by Huff et al. (1944, 1945) has demonstrated tissue stages in bird plasmodiasis; the names *cryptozoite* and *metacryptozoite* have been suggested for these stages. It may be assumed that some similar development takes place in human malaria. There is every evidence that the parasites have changed morphologically and increased in numbers during their absence from the blood, the so-called *tissue* or *exo-erythrocytic* phase. Raffaele (1946) believes he has demonstrated exoerythrocytic forms from the bone marrow in all three of the important species of malaria in man. Parasites can be demonstrated, by transfusion, in the blood a few to several days



Life cycle of malarial parasites.

before the onset of clinical symptoms—before the termination of the clinical incubation period.

Parasites are first found in the stained blood smears shortly before or after the development of clinical symptoms. The youngest form recognized is the young *trophozoite*. This is usually called a "signet-ring" form; it is made up of peripheral cytoplasm, a chromatin dot or nucleus, and a large central vacuole. The trophozoites are within the red blood cells in which they grow and on which they feed. After a definite period of growth and development the nucleus of the trophozoite undergoes division, the parasite then being known as a *schizont*. When maximum nuclear division has occurred, cytoplasmic fission follows; the red cell then contains a mature schizont, sometimes called a segmenter, made up of a number of new, individual parasitic cells identified as *merozoites*. During this period of growth and development, *pigment* is produced and accumulates in amounts varying with the species; as the schizont matures, the pigment granules become clumped into a single mass.

The asexual cycle is completed when the red blood cell ruptures, releasing the brood of merozoites, the pigment, and the remnants of the cell into the circulation. The merozoites immediately attach themselves to new red blood cells which they apparently penetrate although this invasion has not been observed. The cycle is repeated at intervals which vary with the species, continuing until checked by therapy or the reaction of the host.

After the asexual cycle has become established, *gametocytes* may be found in the blood smears. In response to some unknown stimulus, certain schizonts become modified biologically so that their resulting merozoites and subsequent trophozoites do not undergo schizogony. Instead, the merozoites appear in new blood cells as modified ring forms and proceed in their development to the formation of cells that will be differentiated sexually. In theory, all the pre-gametocyte forms (merozoites) from a single schizont become cells with the same sexual characters —either all males or all females, *microgametocytes* or *macrogametocytes*. Thus all merozoites contained in a single schizont may have any one of three destinies: (1) to become trophozoites that will continue schizogony; (2) to become microgametocytes; or (3) to become macrogametocytes. It must be understood that the sexual forms are "mother cells" capable of sexual function only after leaving the human host and further development into male and female *gametes*. Authorities do not agree upon the period of time required for the development of a gametocyte from a merozoite. Since a well-developed gametocyte and a mature schizont of *Plasmodium vivax* may be found in the same red cell, it might be postulated that the time required for their development was the same in this species. However, there is also evidence that some gametocytes require at least twice as long as the asexual forms (Black, 1945).

Cycle in the Mosquito. When an individual with a clinical case or one who is a carrier of malaria is bitten by a female anopheline, parasites are taken up with the blood. The mosquito becomes infected when both micro- and macrogametocytes are present in adequate numbers; the forms of the asexual cycle soon dis-

integrate within the mosquito. The ingested sexual forms become altered and are then known as *microgametes* and *macrogametes*. The microgametocyte undergoes a development known as *exflagellation* during which its nucleus divides, each part taking a filament of cytoplasm. These microgametes, four to eight in number, break away from the mother cell and seek out the female gamete. The macrogametocyte prepares for fertilization by extruding part of its nucleus. A single microgamete penetrates the macrogamete and the nuclei of the two fuse—*syngamy*. A new cell, the *zygote,* is thus formed; it becomes elongated and capable of movement and is then known as the *oökinete*. The motile oökinete migrates through the stomach wall of the mosquito, coming to rest between the epithelium and the outer elastic membrane. A wartlike growth, the *oöcyst,* develops, and within it the nuclear material is divided and reorganized—*sporogony*. The fully developed oöcyst ruptures and releases thousands of *sporozoites* into the body cavity of the mosquito to penetrate tissues and fluid. Those that reach the salivary glands may be transmitted to the next, intermediate host.

The time required for the completion of the sexual cycle, from ingestion of gametocytes to the appearance of sporozoites in the salivary glands, varies in the three common species of plasmodia that may be found in man. Environmental temperature is also an important factor, the development of gametes and sporogony being retarded by low temperatures and inhibited by those below 15° C.; matured sporozoites will survive at lower temperatures. The time required for the development of *Plasmodium vivax* and *P. falciparum* is about the same; *P. malariae* develops more slowly. Under optimum conditions, *P. vivax* may complete the sexual cycle in about 10 days. There may be few oöcysts present, or many, maturing at the same time or in different stages of development. The mosquito may harbor more than one species and is not affected by the presence of the parasites.

Prevention and Control of Malaria. An appraisal of the malaria problem is desirable in a given region before control is initiated. This is approached by a study of the insect vectors and the reservoir host. In highly endemic areas, adult mosquitoes may be captured and examined for oöcysts, the *stomach index* (percentage of positives), or for sporozoites in the salivary glands, the *sporozoite index*. Blooded mosquitoes may be examined by precipitin tests to determine the species preference for animal hosts. Blood smears from a sample or from all of the local population will give the *parasitic index* (percentage positive), and examination for spleen enlargement will give the *splenic index*. These two findings may be combined to give the *endemic index* (the number of persons with enlarged spleens plus the number of those with normal spleens but showing parasites in the blood, reduced to a percentage of the total number examined). Age is an important factor, children serving as a better reservoir than adults.

The control of malaria is an involved problem and comprises a special field in itself. In addition to destruction and control of the mosquito host (see Medical Entomology), protection from bites by anophelines, especially by those potentially infected, is of most importance. As an adjunct, the parasites may be eliminated in carriers by appropriate therapy.

The use of atabrine as a suppressive drug during World War II was accompanied by a dramatic reduction in the incidence of disability due to malaria. With the recommended dosage of 0.1 Gm. daily (given over long periods of time without evidence of toxicity or serious incompatibility) clinical illness and the accompanying infectivity for mosquitoes were almost completely eliminated. Recent work indicates that the new synthetic, chloroquine, is superior to atabrine as a therapeutic agent and probably as a prophylactic drug (Most et al., 1946). A still later drug, paludrine, has enormous possibilities since it is the first causal prophylactic to be synthesized which is effective in non-toxic doses. It has had extensive tests (Adams et al., 1945; Maegraith et al., 1945; Fairley et al., 1946) and probably will have its greatest use against relapsing vivax malaria and in malaria control.

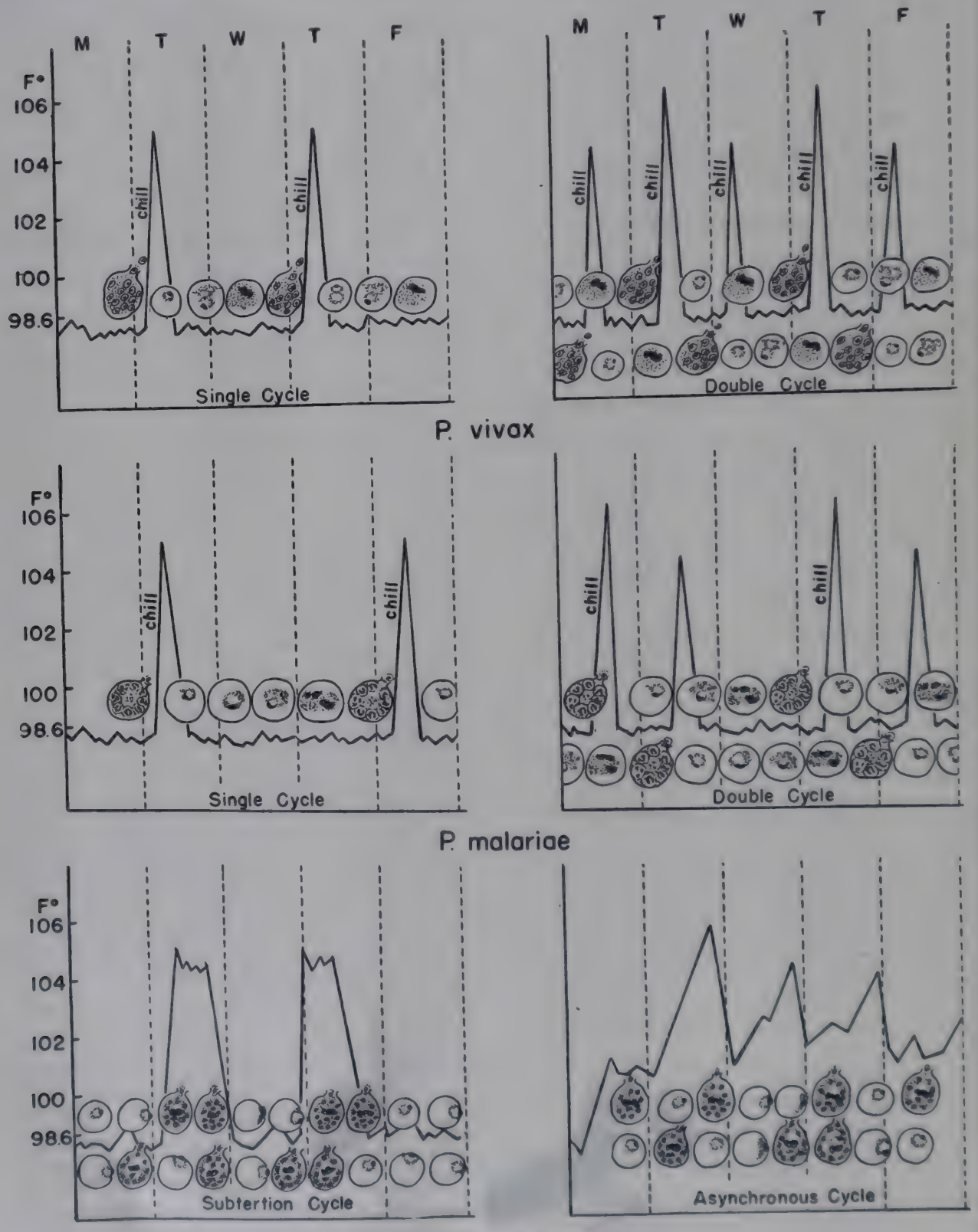
Clinical Illness. Infection in man may be initiated by the inoculation of sporozoites or by the injection of asexual forms. The former is usually mosquito-transmitted and is referred to as sporozoite-induced malaria; James, Nicoll, and Shute (1927) reported the use of suspensions of sporozoites for artificial infections, and this procedure has been employed in experimental malaria as more nearly duplicating natural infections. When blood containing the asexual forms is injected into man, we speak of trophozoite-induced infections. This may be an accidental transfer as seen in drug addicts or following blood transfusion; it is the method commonly used when malaria is employed as a therapeutic agent; and it is the only procedure by which *Plasmodium knowlesi* has been transmitted.

The incubation period in sporozoite-induced infections is fairly constant for each species. There is usually a period of two weeks between the mosquito bite and the onset of clinical symptoms in vivax infections; it is occasionally a few days shorter and may be a few days to many weeks longer. The incubation period for *Plasmodium falciparum* infections is a few days shorter than for those of *P. vivax,* while *P. malariae* takes about twice as long. These periods are only slightly shortened by heavy sporozoite inoculations, suggesting that the tissue phase cannot be hurried. On the other hand, the period between injection of trophozoites and onset of symptoms is generally proportional to the number of parasites introduced; it may be shortened or almost eliminated by transfusion with heavily infected blood.

The rapidity with which parasites may increase in the blood is determined by the number of merozoites produced by each schizont and the frequency with which schizogony is completed. Certain species have an affinity for red cells of certain age and this, together with the natural defenses of the body, may restrict the maximum number of parasites circulating in the human host.

	P. vivax	*P. malariae*	*P. falciparum*
Length of asexual cycle	48 hours	72 hours	36–48 hours
Average number of merozoites	16	8	20
Age of cells preferred	Young	Old	All ages
Usual number of parasites	20–40,000	5–10,000	50–100,000
Maximum (per cu. mm.)	50,000	20,000	One-third of red cells

All phases of the clinical illness in man are related to the asexual cycle and the asexual forms of the parasites; gametocytes do not produce symptoms and disappear if not picked up by the appropriate mosquito. The classical phenomena of malaria are expressed in a paroxysm consisting of chills, fever, and sweating; these symptoms are initiated by the rupture of cells containing mature schizonts. Since the schizonts tend to mature simultaneously, the clinical paroxysms are



Fever charts in malaria.

spaced according to the length of the asexual cycle and give rise to common names for each species:

Plasmodium vivax: Tertian malaria; paroxysms on the first and *third* days, every 48 hours; also known as *benign* tertian because the untreated infection usually terminates in a few days or weeks without serious effects on the host. The specific name refers to the active (vivacious), ameboid movement of the growing parasite.

Plasmodium malariae: Quartan malaria; paroxysms on the first and *fourth* days, every 72 hours. The specific name does not reflect the importance of this species today; in common usage, "quartan malaria" is preferred to "malariae malaria."

Plasmodium falciparum: Subtertian malaria; paroxysms occurring with *less than tertian* periodicity; also known as *malignant* tertian (often fatal), tropical, or estivo-autumnal malaria. The specific name was assigned because of the characteristic shape of the gametocytes produced.

It so happens, more often than not, that parasites do not become organized into a single group maturing at the same time. It is possible to have two groups of *Plasmodium vivax* maturing on alternate days, a *double cycle,* resulting in a quotidian fever instead of the tertian type. The parasites of *P. malariae* may fall into one, two, or three groups, the clinical course being influenced accordingly; a double cycle results in paroxysms on two days out of three, and the triple cycle in daily paroxysms. The falciparum parasites may never fall into groups; when they do become grouped the period of rupture is not a sharply defined time and the clinical reaction is a prolonged fever rather than a sudden chill and abrupt rise and fall in temperature.

The clinical reaction may be provoked by relatively small numbers of parasites, especially in the primary attack; the blood smears may disclose no organisms until multiplication has continued for another cycle or two. The severity of the early paroxysms is proportional to the number of parasites present in a given individual; the first rise in temperature may be slight but increasing with each successive cycle of schizogony. When the number of parasites is sufficiently high to produce a moderate to severe fever the temperature rise may be accompanied by a chill.

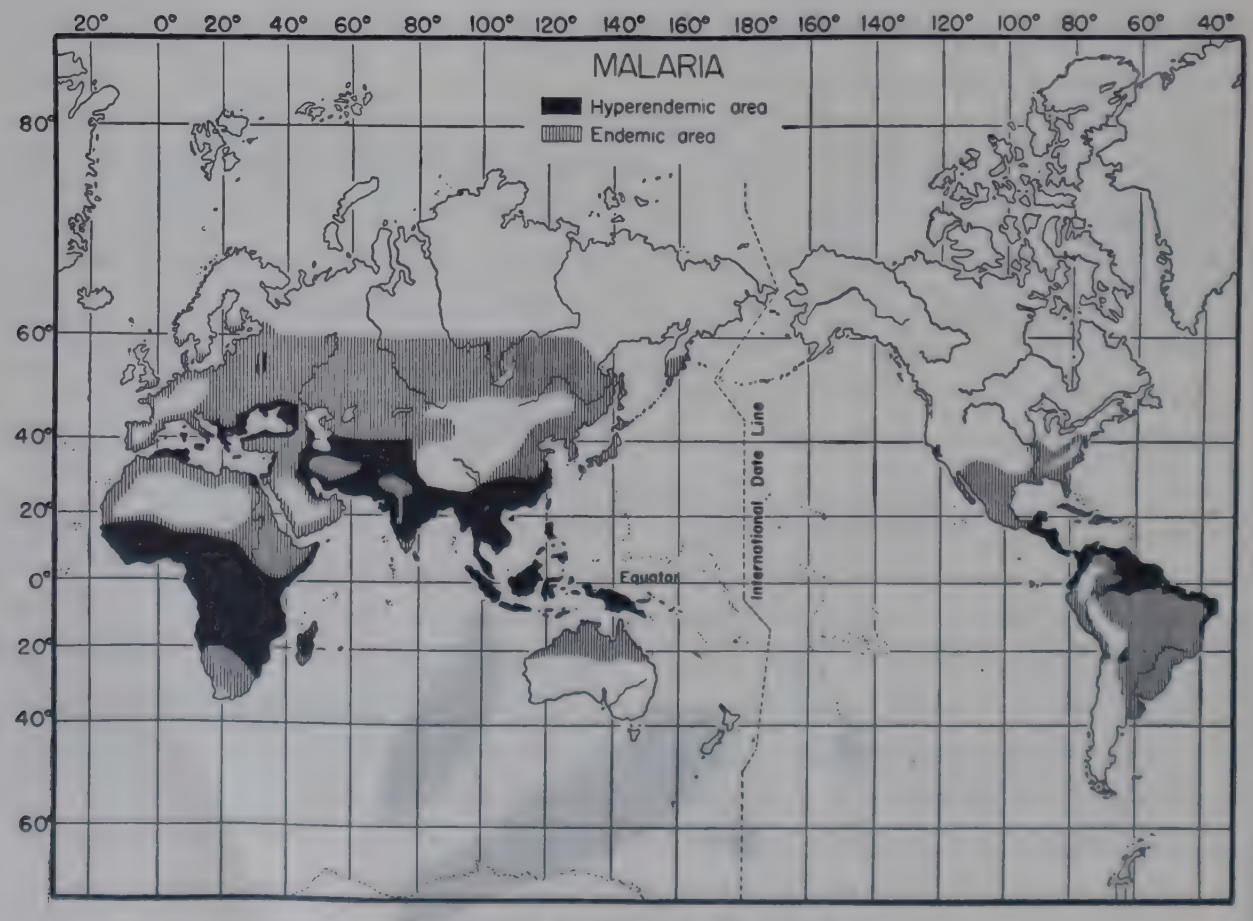
Schizogony of *Plasmodium falciparum* differs from that of the other plasmodia in human beings; the blood cells containing young trophozoites circulate freely in the blood stream for only a few hours. These infected cells become "sticky" and tend to adhere to other cells and to the capillary walls in internal organs. In these capillary beds, older trophozoites become schizonts, and merozoites are liberated to invade new red cells which may again circulate for a few hours. The agglutinated cells and the plugging of the capillaries result in focal symptoms and local pathologic lesions (cerebral malaria) which may produce a fatal termination. In addition, the parasites of *P. falciparum* are not readily controlled by the host and may invade any red cell, increasing numbers also leading to a fatal outcome. The condition of the patient with parasites numbering 200,000 or more per cu. mm. blood must be considered critical and may not improve. Anoxia may play

an important part in death from falciparum malaria (Rostorfer and Rigdon, 1945).

Mixed Infections. The presence of two or more species of plasmodia in the same individual is called a mixed infection. When *Plasmodium falciparum* and *P. vivax* are introduced at the same time, parasites of the former dominate the clinical and laboratory pictures. When the falciparum attack subsides, *P. vivax* may appear and produce subsequent relapses. In endemic areas, persons with chronic malaria may show all of the three common species in a single blood smear; mixtures of *P. vivax* and *P. falciparum* are most common.

Immunity. Immunity to malaria may be expressed by failure of infection to produce illness, with or without parasitemia; by a mild illness following infection; or by clinical symptoms of relatively short duration. Such immunity is usually temporary; it is believed by some to be lost when parasites have been completely eliminated from the body of the host. The Negro race is considered to be partially immune to vivax malaria in the United States although colored troops exhibited a susceptibility equal to that of whites when exposed to the strains of *Plasmodium vivax* encountered in the Pacific area (Sapero and Butler, 1947). The Negro is also said to be somewhat immune to infection with *P. knowlesi*. Infection by a single strain of *P. vivax* may result in immunity to that one strain with partial immunity to other strains of the same species. There is apparently no cross immunity between the different species.

Blackwater Fever. This is a clinical disease entity usually associated only with falciparum infections. It is seen following repeated attacks or reinfections. The



Geographic distribution of malaria.

exact circumstances producing this condition are unknown; various precipitating causes and contributing factors have been suggested. For the laboratory investigation, parasites may be present in the blood specimens before and after the attack but usually are not found during the acute episode. There is intravascular hemolysis resulting in a marked reduction in red cells. The released hemoglobin is excreted in the urine, giving the disease its descriptive name. Albuminuria is consistently present while red cells are rare or absent. Several excellent articles have recently appeared summarizing our present knowledge of this disease (Kitchen and Sadler, 1945; Maegraith, 1946).

TERMINOLOGY. Certain terms have come into common usage in malaria and have recently been defined by a committee of the National Research Council (1945):

INFECTION. Infection is the condition in which living parasites are within the body, demonstrable by any laboratory method or clinical expression.

PARASITEMIA. This is the condition in which parasites are in the blood; when they can be demonstrated by blood smear the parasitemia is patent; otherwise it is subpatent.

PREPATENT PERIOD. The prepatent period is the interval between the introduction of parasites and their first appearance in blood smears.

PATENT PERIOD. The patent period is any period during which parasites can be demonstrated in the blood smear.

CLINICAL ATTACK. The clinical attack is a phase of the disease marked by clearly defined symptoms due to malaria parasites. The first attack after infection is the primary attack; if late in appearing, it is a delayed primary.

LATENCY. Latency is any state in which infection is hidden.

RELAPSE. This may be either clinical or parasitic; the clinical relapse is any clinical attack after the primary one; a parasitic relapse is the reappearance of, or increase in, circulating parasites.

CURE. Cure may be clinical (freedom from attacks), parasitic (eradication of infection), spontaneous, or chemotherapeutic.

COMPLETE PROPHYLAXIS. Complete prophylaxis is prevention of parasitemia by action upon the sporozoites or subsequent stages of the parasite.

PARTIAL PROPHYLAXIS. Partial prophylaxis is delay in parasitemia.

SUPPRESSION. This is a limitation or reduction of infection without eradication. It is usually accompanied by prevention of clinical symptoms.

Diagnosis of Malaria in Man. In areas of endemic malaria, vivax and falciparum infections are those most frequently encountered; the former may be more important elsewhere because of the recurrent nature of this disease. Returning military personnel of World War II have shown vivax infections almost exclusively, those due to *Plasmodium falciparum* having died out during the period of suppressive treatment. Quartan malaria tends to be localized in its distribution and is of secondary importance. However, it is capable of long periods of latency; the reported cases of malaria following transfusions with blood from donors who had their last malaria attack 10 or 20 years previously have all been quartan. Ovale

Caption for Plate VI

Benign Tertian Parasites

(1) Normal red cell for comparison of size.
(2) Trophozoite, young ring form.
(3) Trophozoite, full grown. Red cell is enlarged and Schüffner's dots are present.
(4) Schizont, young form, undergoing second nuclear division.
(5) Schizont, quarter grown. Nuclei composed of fine chromatin granules in irregular clumps. Yellowish brown pigment is present.
(6) Schizont, mature form. Nuclear division complete. Cytoplasm dividing preparatory to liberation of merozoites.
(7) Macrogametocyte (female gametocyte). Cytoplasm is blue, chromatin eccentric, compact, deep red, and surrounded by a halo.
(8) Microgametocyte (male gametocyte). Cytoplasm is greenish blue, chromatin central, diffuse, and light red.

Quartan Parasites

(1) Trophozoite, young ring form. Fine black pigment granules are present.
(2) Trophozoite, young band, or equatorial form.
(3) Trophozoite, a more mature oval form, showing beginning nuclear division.
(4) Schizont, young, binucleate form, heavily pigmented.
(5) Schizont, older band form. Pigment is more abundant about periphery.
(6) Schizont, mature. Chromatin clumps form eight nuclear masses arranged around a central mass of pigment.
(7) Macrogametocyte (female gametocyte). Chromatin is compact and deep red. Pigment abundant.
(8) Microgametocyte (male gametocyte). Chromatin is diffuse and pale. Pigment abundant.

Malignant Tertian Parasites

(1) Trophozoite, young, hairlike ring form.
(2) Trophozoites, young ring forms. Characteristic multiple infection of a red cell showing also peripherally placed forms.
(3) Trophozoite, full grown. Rarely seen in peripheral blood except in very heavy infections.
(4, 5, 6) Schizonts in successive stages of maturity. Rarely seen in peripheral blood. Specimens from red cells in a brain capillary of a fatal case of cerebral malaria.
(7) Macrogametocyte (female gametocyte). Shows characteristic crescent shape. Nucleus is compact, deeply stained. Pigment clumped in center.
(8) Microgametocyte (male gametocyte). Chromatin is pale staining and diffuse. Pigment is dispersed.

Caption for Plate VII

Plasmodium vivax in thick smears stained with Giemsa, using a buffer of pH 6.8.

(*Upper left*) Smear made at the height of the malarial paroxysm, showing a mature schizont with approximately 14 merozoites, a recently ruptured schizont, and a number of young nondiagnostic trophozoites. A segmented neutrophil is seen in the upper portion of the field.

(*Upper right*) Smear made about 6 to 8 hours after the paroxysm, showing young trophozoites, some of which are beginning to exhibit the ameboid cytoplasm diagnostic for *P. vivax,* and a gametocyte (female). A segmented neutrophil is located about nine o'clock in the field.

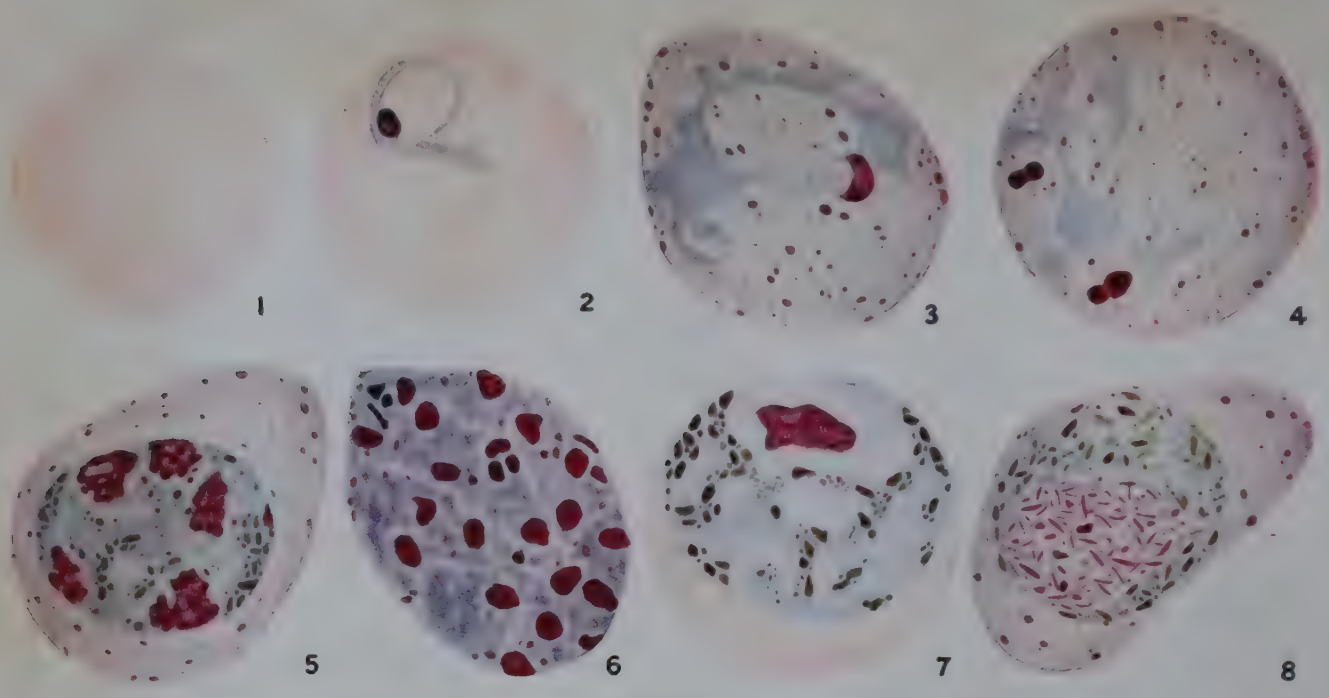
(*Middle left*) Smear made about 20 to 30 hours after the paroxysm, showing the very characteristic ameboid trophozoites of this species. A segmented neutrophil is shown about five o'clock in the field.

(*Middle right*) Smear made about 40 hours after the paroxysm, showing two young schizonts and a gametocyte (male). An eosinophil is seen about one o'clock in the field.

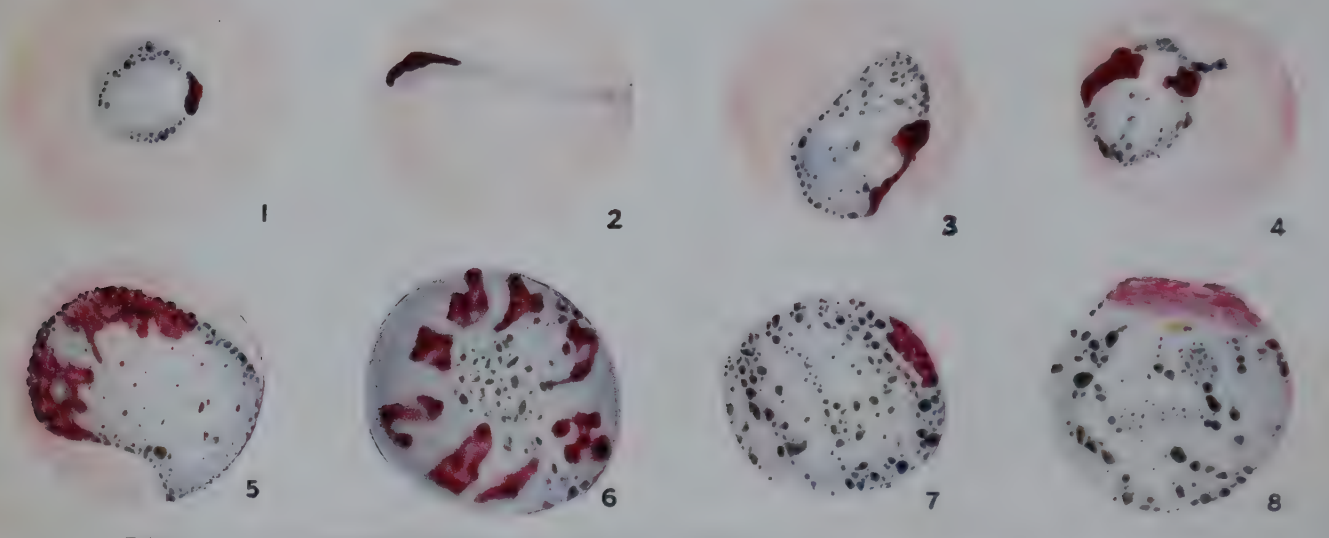
(*Lower left*) Smear made shortly before paroxysm, showing two nearly mature schizonts and a gametocyte (female). A segmented neutrophil is shown about three o'clock in the field.

(*Lower right*) Smear made about 30 hours after the paroxysm. In a fair proportion of *P. vivax* thick smears the parasitized red blood cells fail to lyse as readily as do the unattacked cells, thus giving a very characteristic picture. This is best seen at the periphery of the smear. A segmented neutrophil is depicted at the upper left corner of the field.

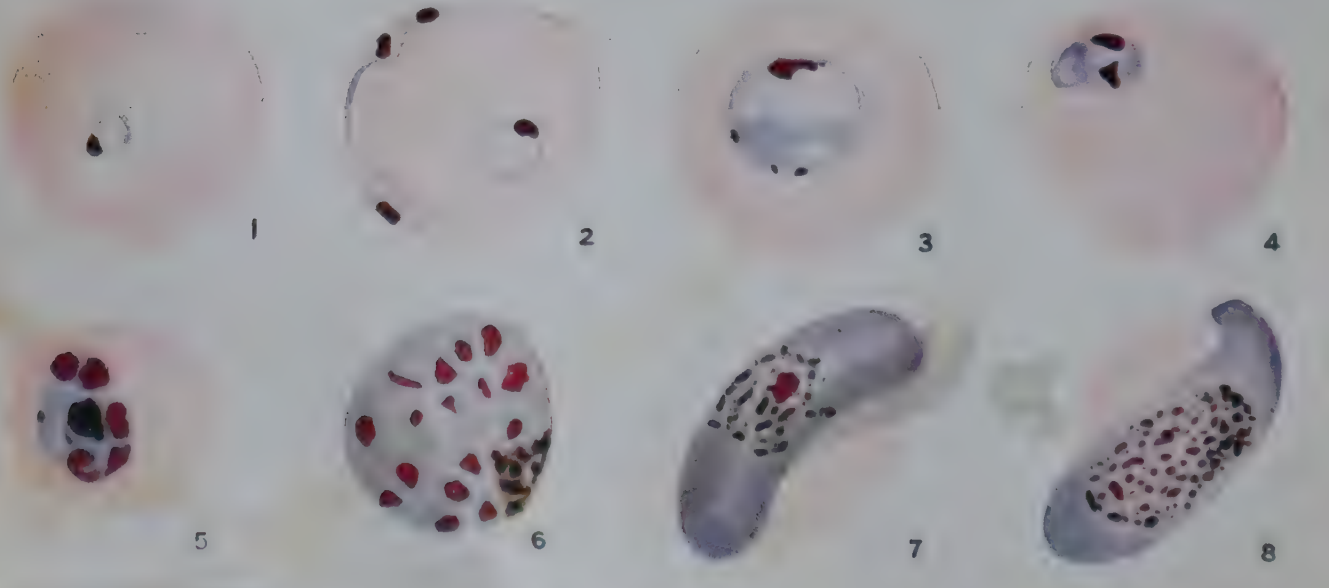
BENIGN TERTIAN PARASITES (*Plasmodium vivax*)



QUARTAN PARASITES (*Plasmodium malariae*)



MALIGNANT TERTIAN PARASITES (*Plasmodium falciparum*)



U. S. Naval Medical School (*A. D. Wallace*)

MALARIA PARASITES

PLATE VI



PLATE VII

(See p. 498 for caption.)

infection is of little significance as a disease entity. *Plasmodium knowlesi* is encountered only experimentally.

A positive diagnosis of malaria is made or confirmed when parasites are demonstrated in a blood or tissue specimen. Although the organisms are visible in fresh blood and were first recognized in this medium, the advantages of stained preparations preclude the routine examination of wet smears. The technician identifies the parasites and the characters of the different species in stained blood smears. The specific diagnosis may be of great importance because of the serious nature of falciparum infections and the tendency for relapses in vivax.

The diagnosis is often made from *thin blood smears*. When parasites are less numerous, and for those who have become proficient in its use, the *thick film* presents definite advantages. Parasites may also be demonstrated as present by inoculating blood into another susceptible person. *Sternal puncture* is sometimes indicated if parasites cannot be demonstrated by examination of blood. This procedure, replacing the hazardous splenic puncture, is reported to be of value in falciparum infections when the parasites are absent from the peripheral circulation.

Stained with the polychrome stains, the parasites are seen to be composed of blue cytoplasm, red chromatin or nuclei, and pigment which varies from greenish yellow to black. The identification of species requires a careful study of all stages available in the preparation. When only a few young trophozoites ("rings") can be found it will be difficult, if not impossible, to make a specific diagnosis; but there are characteristic forms for each species and these are identified before the final diagnosis is established. The older stages of the asexual cycle are more helpful and subsequent examinations may be necessary in order to find them. If the preparation shows no parasites the examination should be repeated at 8- to 12-hour intervals until malaria is eliminated or established as the diagnosis. Recent technics for demonstrating malaria parasites which require further testing are examination of blood by darkfield (Packer, 1945) or fluoroscopic (Metcalf, 1945) microscope, by the preparation of superimposed smears (Perret-Gentil, 1945), or by supravital staining (Chatterjee, 1947).

Use of Adrenalin. Adrenalin has been suggested from time to time as an adjunct in both diagnosis and therapy. In theory, the drug will force parasites into the circulation by contracting the spleen, making them available for microscopic examination or accessible to the therapeutic action of antimalarials. In the recent experience of the Navy, this has been of little or no value. On the other hand, Hunter (1945) reports excellent results with this method.

Therapy and Suppressive Treatment. The rapidity with which antimalarial drugs affect the circulating parasites will depend upon the method of administration and the efficacy of the agent. Atabrine, quinine, chloroquine or paludrine given by mouth in adequate dosage will usually control the clinical manifestations within 48 hours. As would be expected, the circulating parasites parallel the clinical symptoms in disappearance. While in some patients parasites may still be demonstrated up to 96 hours after therapy has been instituted, most patients

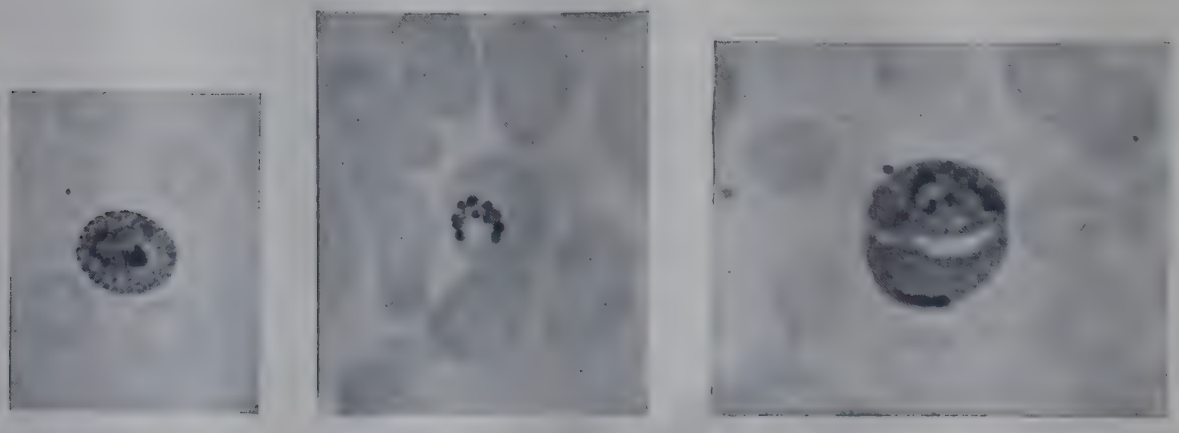
have negative blood smears within 48 hours. It is thus evident that, for several hours after treatment is started, repeat examinations may be useful to obtain or confirm a specific diagnosis. However, if the original number of parasites was low, even small doses of antimalarial drugs may be sufficient to reduce that number to the point that subsequent examinations will not reveal the parasite.

Men who have taken suppressive treatment as recommended by the Navy and Army have shown negative blood smears, although infection was known to be present. Irregular or inadequate dosage with suppressive drugs may permit parasites to circulate in sufficient numbers to be found in smears, with or without clinical symptoms being present. Those who have had repeated attacks may develop sufficient immunity to have circulating parasites, demonstrable by blood smear, with no clinical symptoms when suppressive treatment is stopped.

Plasmodium Vivax in Thin Blood Smears. The sprawling shape of the older forms in the stained blood smears is the most constant, outstanding feature of this species. At the end of the incubation period the blood will contain both young and old asexual forms at any one time; with subsequent paroxysms, most of the parasites become synchronized in their development so that a single examination will show stages corresponding to the phase of the cycle. However, there are always some parasites that are "out of step" with the majority, making it possible to find all forms in any smear, a second diagnostic feature.

The "rings" usually are not characteristic; if they must be utilized for diagnosis, they are heavier and larger than those of *Plasmodium falciparum*. It is not unusual to find more than one parasite in the same red cell and as many as three and four have been observed. Ring forms are occasionally found with two nuclei, these usually being unequal in size and having the appearance of fragments. Older trophozoites (after 8 to 12 hours) show the characteristic ameboid cytoplasm and produce specific changes in the infected cell: it becomes pale in color and larger than normal. Stippling of the infected cell, known as Schüffner's granules, appears about the same time, becoming more marked as the parasite grows. These granules are not always seen but may be accentuated by overstaining.

When the asexual cycle is three-quarters completed, at about 36 hours, the



(*Left*) Trophozoite of *Plasmodium ovale*. (*Center*) Schizont of *Plasmodium falciparum*. (*Right*) Gametocyte and schizont of *Plasmodium vivax* within the same red cell. (Photomicrographs (*left*) × 950, (*center* and *right*) × 1200.)

nucleus of the trophozoite divides for the first time. The parasite has then grown to be as large as the normal red cell, or larger. Successive divisions follow rapidly until maximum division (12 to 24 daughter nuclei) has occurred. Scanty granules of pigment appear in the cytoplasm of the older trophozoites, becoming clumped in an eccentric position in the mature schizont.

Gametocytes may be found at any time during the course of vivax infections, usually appearing during the first few days of illness. They are rounded in outline, as large as or larger than the normal red cell, and show heavily pigmented cytoplasm with a single chromatin mass. Immature forms, less heavily pigmented, are easily confused with old trophozoites. The red cells containing them are enlarged and stippled. Recognition that these forms are gametocytes is essential so that the diagnosis will not be confused with quartan in which the asexual stages are heavily pigmented.

Plasmodium Malariae in Thin Blood Smears. The outstanding features in this species are the compactness of the parasites and their heavy pigmentation. The infected red cell is not altered in size or appearance; while granules (Ziemann's) have been described, they are seen only after special staining. All forms of the asexual cycle may be found in a single blood smear. Again, the rings are of little help in species diagnosis; of the three common species, they are middle-sized. The growing parasites show little ameboid activity, the cytoplasm remaining compact instead of sprawling. This compactness results in deeper staining of the cytoplasm in the fixed smears.

The growing trophozoite and young schizonts may be seen stretched out across the red cell in a "band" formation; this is characteristic of this species although it may be encountered in growing trophozoites of *Plasmodium falciparum*. Pigment appears early in the trophozoite stage and is coarser and darker than that found in *P. vivax*. Again, the trophozoite grows for about three-quarters of the 72-hour cycle before the nucleus divides. The mature schizonts show 6 to 12 nuclei, often arranged in a geometric pattern with the clumped pigment centrally placed, the so-called "rosette" or "daisy head."

The gametocytes are usually found later in the course of the disease than is seen in *Plasmodium vivax*. Round or oval in outline, they are readily confused with old trophozoites because of the heavy pigmentation of these asexual forms. Pigment in the gametocytes is more likely to appear as rods or bars rather than as granules or clumps. The sexual forms are smaller than the corresponding ones in *P. vivax*.

Plasmodium Falciparum in Thin Blood Smears. The outstanding feature of this species is the presence of rings only or of rings and gametocytes in the usual blood specimen. While the sexual forms, often called "crescents" because of their shape, differ markedly from those of all other species, they do not appear in the blood until about 10 days after the onset of fever and are of little or no help in the diagnosis of the acute illness. When rings and crescents are both present the diagnosis of clinical illness must still be made from the asexual forms; the young trophozoites may belong to another species, a mixed infection being present.

The absence of all maturing asexual forms is the first suggestive character in this species; however, any heavy infection may show an occasional schizont and all asexual stages may be present in overwhelming parasitemia. The very young trophozoites are delicate and smaller than those of vivax and quartan infections. Double-nucleate forms are frequently seen, the nuclei being symmetrically placed and smooth in outline rather than broken. Two, three, or more parasites are very often found in the same red cell, the frequency being determined in part by the total number of parasites present. The chromatin nucleus has been described as lying outside the ring of cytoplasm in contrast to *Plasmodium vivax* where it is in or within the circle. The young parasites often appear to be attached to the external surface of the red cell, giving rise to the descriptive terms "marginal," "accolé," and "appliqué." While the infected cell is not enlarged, red and irregularly shaped dots called "Maurer's spots" are very occasionally seen, again emphasized by overstaining.

About the time the trophozoites begin to lose their ring shape the infected red cells are lost from the circulation. Older forms found in placental smears, in material obtained by biopsy or at autopsy, or rarely in blood smears show the early presence of fine granules of black pigment. The schizonts are small with delicate nuclei and a dark, prominent pigment mass; they may resemble those of quartan, being differentiated by the number and size of the merozoites. The gametocytes have been variously described as sausage-shaped, banana-shaped, and crescentic. Their centrally placed nucleus is surrounded by pigment granules.

Plasmodium Ovale. This species was named from the oval or tear-drop shape of the infected blood cells. The asexual cycle is completed in 48 hours, and the infection in man is reported as more benign than vivax. Because of resemblances to vivax and quartan parasites in the blood smears, it may be necessary to follow the complete asexual cycle, taking repeated specimens. The infected blood cell is enlarged and shows a granulation similar to Schüffner's, possibly coarser and darker staining. The growing parasites show ameboid activity and sprawling cytoplasm. The mature schizont resembles that of quartan in having 6 to 12 nuclei, but it is a larger parasite. The gametocytes cannot be differentiated from those of *Plasmodium vivax*.

Plasmodium Knowlesi. While this parasite is not likely to be encountered without previous knowledge of the species involved, a few characters may be listed. The trophozoite shows a nucleus which appears to be surrounded by a "halo." Band forms are frequently found. The mature schizont contains 6 to 10 merozoites, usually 8 or 9.

Gametocytes. The sexual forms are of little assistance in making a species diagnosis. Immature gametocytes, closely resembling old trophozoites, are only occasionally found in blood smears, usually developing in capillaries of the internal organs. At times it may be necessary to differentiate between the microgametocyte and the macrogametocyte of the same species, primarily where infectivity for mosquitoes is being studied. The important differential features may be summarized as follows:

Microgametocyte (Male)	*Macrogametocyte (Female)*
Smaller in size.	Larger in size.
Chromatin diffuse, pale, centrally placed.	Chromatin compact, more deeply stained, often eccentric.
Cytoplasm pale, sometimes greenish, often almost colorless.	Cytoplasm stained a more definite blue.

Summary of Diagnostic Features in Thin Smears. The diagnosis of the three common species of malaria may be reached by answering a series of questions as the stained smear is examined:

Does the growing parasite enlarge the infected red cell?
Are granules or stippling present in the infected cell?
What stages of the parasites are found? Are gametocytes present?
How frequently is there more than one parasite in the same red cell?
What is the maximum number of nuclei found in the schizonts?
How often do you find double-nucleate rings?
What is the outstanding feature of the cytoplasm? Sprawling? Compact? Bands?
How early does pigment appear and what is its color and amount?

Key to Human Plasmodia in Thin Smears

1. Only very young ring trophozoites present; no enlargement of the infected cell; no stippling of the infected cell ..... *Plasmodium* species*
   Older forms of the asexual cycle or gametocytes present 2
2. Parasitized red blood cell enlarged; *or,* parasite with markedly ameboid cytoplasm; *or,* infected cell with Schüffner's granules; *or,* schizont larger than normal red cell and with more than twelve nuclei ..... *Plasmodium vivax*
   Without any one or any combination of the above features 3
3. Mature schizont with 6 to 12 nuclei arranged in "rosette" or "daisy-head" pattern; *or,* trophozoite with compact, heavily pigmented cytoplasm; *or,* with pigmented equatorial-band trophozoites or young schizonts ..... *Plasmodium malariae*
   Without any one or any combination of the above characters and, in addition, crescent-shaped gametocytes present; *or,* parasitized red blood cells showing Maurer's spots ..... *Plasmodium falciparum*

Thick Blood Films. The thick blood film is a preparation in which a relatively larger quantity of blood is placed on a small area of the slide. The hemoglobin of

*In such slides the technician can never be sure that any report other than "positive for malaria parasites" is correct. The commonly accepted procedure is to make such a report and request an additional smear to be made in about eight hours. Treatment started immediately will have no appreciable effect in that length of time. If the parasites of the later smear show no development, a diagnosis of *Plasmodium falciparum* is justified. In addition, certain other features give strong evidence that *P. falciparum* is present. These may be called percentage characters—they may and do occur in other species, but much less often than in *P. falciparum*. An observation of one feature is interesting, two make a strong impression, and three or more almost a certainty. These characters are: (1) presence of a large number of parasites and only ring forms; (2) multiple infection of the red blood cell; (3) double-nucleate trophozoites where both nuclei are round and neither appears to be a fragment; and (4) "appliqué" or marginal parasites.

the red cells is removed in the staining procedure, rendering the film transparent. The two principal structures of the parasite, the red nucleus or nuclei and the blue cytoplasm, must be seen before identifying an object as a parasite. The nucleus stands out prominently and appears more compact than in the thin smears. The cytoplasm varies in shape and shade of blue according to the species and the stage of development. As the rings of young trophozoites collapse, the cytoplasm forms a tail of blue which may or may not be visibly attached to the chromatin. Rarely the ring form will persist, sometimes appearing as a broken ring but more commonly as a blob or dash of blue. Various descriptive terms have been applied: "swallow" form when there is a dash of blue on each side of the nucleus; "comma" form when the cytoplasm becomes a curved thread; and "flagstaff" form when there is a dash of blue extending from a red dot. The delicate cytoplasm of *Plasmodium falciparum* may be barely visible and often appears to be less in amount than the nucleus. The pigment has the same appearance as in thin smears; white cells containing pigment and having diagnostic importance are occasionally seen. (See Plate VII.)

The differential diagnosis of species is best made from the older asexual forms; diagnostic features and characters will usually be found when many parasites can be observed. Shadows or skeletons of red cells and Schüffner's granules may be found occasionally in vivax infections. Young trophozoites are no more diagnostic than they are in thin smears. *Plasmodium vivax* and *P. malariae* rarely, if ever,

Table 53

SUMMARY OF CHARACTERS IN THICK BLOOD FILMS

	P. vivax	*P. malariae*	*P. falciparum*
Stages of development usually present	*All forms*	*All forms*	*Only rings* or rings and crescents
The young trophozoites	Largest of the three species; heavier chromatin dot; cytoplasm in strings and blobs	Medium-sized; nucleus may be as large as vivax; cytoplasm compact, close to nucleus	Smallest of the three species; small chromatin dot, *frequently double;* cytoplasm delicate, close to nucleus
The older trophozoites	Masses and blobs of light-blue, *sprawling cytoplasm,* often arranged in triangle and "Y" forms	A single blob of *compact, deep-staining cytoplasm;* heavily *pigmented;* usually rounded	When found, very much like young vivax; older forms usually absent; may show double nuclei; fine, dark pigment
Schizonts	Irregular in outline; *12 or more nuclei* when mature; pigment yellow to brown, eccentric	Regular in outline; *12 or less nuclei* when mature, often in *rosette* form; pigment dark brown, central	When seen, small in size of parasite and nuclei; pigment prominent, dark to black
Gametocytes	Spherical, pigmented; similar to old trophozoites	Spherical, pigmented; similar to old trophozoites	*Sausage-shaped* or crescents

show only rings so that observation of many parasites becomes important. When difficulties are encountered in species diagnosis the margins of the blood film should be searched; here the parasites are less distorted and resemble their appearance in the thin smears. Gametocytes may be more confusing than helpful; crescents may be seen on end or folded over and may be mistaken for trophozoites or schizonts of *P. malariae*. Vivax or quartan macrogametocytes cannot be differentiated from the mature trophozoites of these species; the microgametocytes are distinctive in their relatively large nuclei, more deeply stained than in the thin smear, surrounded by light-staining or colorless cytoplasm containing the typical pigment granules.

Fallacies. Various objects may be mistaken for parasites in the stained preparations. Blood platelets, especially when overlying a red blood cell in the thin smear, are the cause of many errors. All thick films will show platelets, debris, and the distorted nuclei of white cells. Rounded objects may take a red tint from the stain and, to add to the confusion, may be on or near a mass of blue. Dust particles, spores, bacteria, and other artefacts may reach the microscopic field through dirt on the skin, unclean slides, or contaminated water. An object should not be called a parasite if it can be interpreted as an artefact. Unless the parasite is very definite, others should be seen before a positive diagnosis is given.

Concentration and Cultivation. Attempts to concentrate the plasmodia in human blood have not been attended by any great degree of success. The Bass-Johns technic (1912) which depended upon centrifugation and recovering parasites from the bottom of the leukocytic layer has been modified by laking the blood and spinning down the parasites with the cellular debris. This latter method has been utilized in attempts to concentrate parasites for vaccines and preparation of antigens. For diagnostic purposes, no concentration technic surpasses the thick blood film.

Recent attempts at cultivation of the malaria parasites have shown more promise than the former method of Bass (1912). By carefully controlling environmental conditions, Ball et al. (Report to the National Research Council, 1944; 1945) have succeeded in carrying parasites through several asexual cycles; they have been partially successful with human species. The value of such research projects lies in the information to be obtained regarding the physiology and metabolism of these parasites. *P. falciparum* has recently been cultured in serum (Black, 1945). There are also reported attempts, some successful, in cultivating the exo-erythrocytic stages of bird malaria in tissue cultures (Hawking, 1944, 1945).

Complement Fixation. The presence of antibodies in malaria has been demonstrated and repeated attempts have been made to utilize these for diagnostic purposes. Coggeshall (1938) obtained a positive complement-fixation reaction using *Plasmodium knowlesi* parasites as antigen. Other investigators have utilized material obtained from bird plasmodia and from the human species. The reaction appears to be group specific, an antigen from the plasmodia of birds or monkeys giving positive reactions with human sera. Positive reactions appear several days after the primary attack and may continue for several months after the parasites

have disappeared from the blood; the titer increases with subsequent relapses. The application of this test to detect latent cases and potential relapses has not been uniformly successful. A cephalin flocculation test has recently given very promising results (Makari, 1946).

Malaria has long been known to cause false positive reactions to the serologic tests for syphilis. Slightly over 7 per cent of the malaria cases have given false positive reactions at the Naval Medical School.

Laboratory Technics. Thin smears are made as for differential blood counts and may be stained with various polychrome or Romanowsky stains. The pH of stain and water used must be carefully controlled to bring out the desirable contrast in colors. Parasites are sought within the red blood cells. An oil-immersion objective and moderately intense, filtered light are essential; a blue filter should be avoided as it may obscure poorly stained nuclei. The beginner will appreciate a 10x ocular, but the experienced worker increases his efficiency by using a 5x or 6x lens. The search for parasites in these smears is slow and tedious when the number of parasites is small. The more experienced technician finds the thick-film examination much more rapid and, with few exceptions, entirely satisfactory for species diagnosis.

In heavy infections the thin smear gives a rapid diagnosis and reveals the morphology of individual parasites; it quickly dries and can be stained in a few minutes. However, it rarely reveals any parasites when the infection is light. In such cases and where many slides are to be examined, the thick film presents definite advantages. Compared with the thin smear, the amount of blood covered per microscopic field may be increased up to 50 times. The sparse parasites are discovered and the time spent per slide is greatly reduced.

Preparation of Smears. Blood is obtained from the finger or ear. The area to be punctured must be clean and dry; any alcohol left on the skin may fix the blood cells and then they will not hemolyze in the thick-film staining. Thin smears made on glass slides are preferable to the coverslip method. In making the thick film, three small drops of blood are placed near one end of the slide and spread by stirring with the corner of another clean slide. The glass slide should touch only the drop of blood, not the skin, to avoid introducing artefacts into the specimen. The resulting film should be about the size of a dime; if too thick, the film will crack and may not stick. Thin smears may be fixed or stained after a few minutes of drying, but the thick film should dry for at least one hour in open air or one-half hour in the incubator set at 37° C. If not adequately dried, the preparation will not stick to the glass slide during staining. Overheating to hasten drying should be avoided as this also fixes the red cells so they cannot be laked. The preparations should be protected from dust and insects, and thick films must be kept flat until dry. Material obtained from bone marrow by sternal puncture is best made into a thin smear and fixed or stained as thin blood smears.

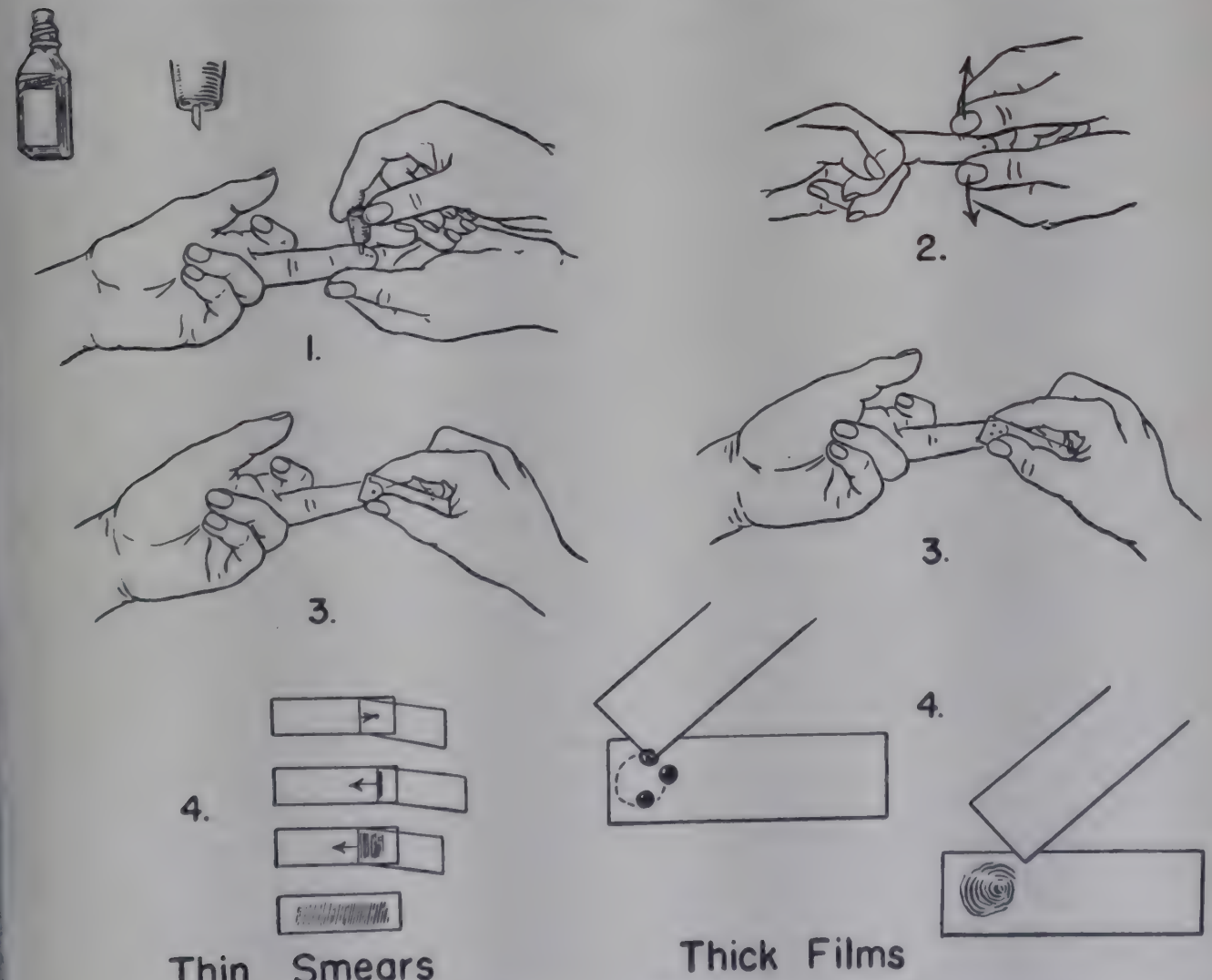
Choice of Stain. Wright's stain is suitable for thin smears and is often selected for routine use because of availability and convenience when only a few slides are being examined for malaria. Giemsa stain is suitable for both thick and thin smears, produces better staining of the parasites, and is more permanent. It lends itself well to use in laboratories that examine large numbers of slides or some slides every day. Wright-Giemsa or Field's stain is suitable for thick films and has the advantage of requiring a shorter staining time.

Buffered Water. Results are more consistently satisfactory if buffered water, pH 6.8 to 7.2, is used for making the staining solutions and as a wash or rinse for stained slides and glassware. Equal parts of anhydrous monobasic sodium or potassium phosphate and anhydrous, or dried U.S.P., dibasic sodium phosphate are thoroughly mixed, the final mixture being adjusted to give the desired pH when 1 Gm. is dissolved in 1 liter distilled water. The salt mixture is kept in small bottles, fresh buffered water being prepared as it is needed. One Gm. (¼ teaspoonful) added to 1 liter of any clean water will produce the desired pH. Heavily chlorinated water should be boiled and cooled before using.

Wright's Stain. This stain is prepared and used in the same manner as for differential blood counts. It may be used for staining thick films if these are first dehemoglobinized in water of proper pH.

Giemsa Stain: PREPARATION. Various commercial firms now prepare a satisfactory Giemsa stain in powder form or in solution. The powdered dye is dissolved in glycerin and diluted with an equal volume of absolute methyl alcohol. In preparing the dye the method reported by Lillie (Public Health Reports, March 12, 1943) is recommended, modified by using 800 mg. of the finished powder for 100 ml. solvent to make the stock solution.

STAINING. The amount of diluted stain needed for the slides on hand or for the day's work is estimated; the stock Giemsa is usually diluted 1 : 30 or 1 : 50 with buffered water. The required amount of undiluted stain is measured in a small graduate and this is poured into the staining dish. The graduate is rinsed with buffered water, and this water and the remainder of the measured diluent are added to the stain in the dish. A dish is selected



Preparation of blood smears.

of such capacity that the blood will be covered with the slide on end. Specimens are left in the stain for about one hour, depending upon preference and experience with the stain. At the end of the staining time, the slides are removed and placed on end in a second dish containing buffered water. Thin smears may be dipped in and out, but thick films should remain three to five minutes. When removed from the rinse, the slides are placed on end on absorbent paper to dry.

The staining time may be shortened by decreasing the dilution used. When speed is necessary for diagnostic purposes, thick-film preparations as little as one hour old may be stained in 5 to 10 minutes in a solution containing 4 ml. stock Giemsa, 3 ml. acetone, and 33 ml. buffered water. This rapid technic is not recommended for routine work. With the usual dilution, 1 ml. stock stain is sufficient for 25 slides. Any remaining stain is discarded at the end of the day. Some workers prefer to lake the thick films for five minutes in buffered water before placing them in the stain; this is especially useful when slides have been kept for some time before staining.

When thin smears are to be stained with Giemsa they must first be fixed by flooding with methyl alcohol. Both thick and thin smears may be made on the same slide. If this is done, care must be taken to protect the thick film while the thin smear is being fixed. Only the thin smear is flooded with methyl alcohol which is washed off with buffered water before the slide is immersed in the staining solution. The thin smear may be made but not fixed or stained, unless desired, until after the thick film has been examined.

Wright-Giemsa (Wilcox, National Institute of Health Bulletin 180, 1942): PREPARATION. Two Gm. Giemsa are dissolved in 100 ml. glycerin by heating the mixture in a water bath at 55° to 60° C. and stirring at intervals for two hours. To this are added 100 ml. Wright's stain solution made with 2 Gm. of powder in 1000 ml. methyl alcohol and aged. After allowing to stand overnight, an additional 800 ml. Wright's solution are added. The preparation is ready to use after filtering.

STAINING. The staining solution is diluted 1 : 9 with buffered water. The dried thick films or fixed thin smears are placed in a staining dish and covered with the diluted stain. After staining for 10 minutes the scum is flushed from the top of the dish; the slides are removed, washed for one minute, and are ready for examination when dry.

Field's Stain: PREPARATION. Two solutions of stain are required: Solution A is made by dissolving 1.3 Gm. medicinal methylene blue and 5.0 Gm. anhydrous disodium phosphate in 50 ml. distilled water. This is boiled and then evaporated almost to dryness in a water bath. To the residue are added 6.25 Gm. anhydrous potassium phosphate, monobasic, and 500 ml. distilled water. After the mixture has been allowed to stand for 24 hours, it is ready for filtering and use. Solution B is prepared by dissolving 5.0 Gm. anhydrous disodium phosphate and 6.25 Gm. anhydrous potassium phosphate, monobasic, in 500 ml. distilled water. After adding 1.0 Gm. eosin the solution is allowed to stand for 24 hours, then it is filtered and ready for use.

STAINING. Thick films need not be so thoroughly dry as when stained by Giemsa. The slides are dipped in Solution A for one to five seconds and then washed in clean water for a few seconds—until the stain ceases to flow from the film and the slide is free of stain. Then they are dipped into Solution B for one to five seconds and again rinsed in clean water for a few seconds. When dry, they are ready for examination.

Precautions. All glassware used for making stain and for staining should be reserved exclusively for that purpose. Glass slides should be clean and free from grease. New slides are preferred, but old slides that have been used only for blood are satisfactory. New slides should be prepared for use by washing in mild soap solution and hot water; they should be rinsed thoroughly in tap water and dried after washing with 95 per cent alcohol. The stock solution of stain should be protected from dirt and moisture. At the end of the day, staining dishes are emptied and rinsed with methyl alcohol, followed by clean water and then buffered water.

Parasite Densities. The number of parasites in the blood may be determined in various

ways. An estimate of the relative density may be obtained by observing the number in the average microscopic field of the thick blood film. If such a procedure is used it must be assumed that the blood films are fairly constant in thickness. The parasites may also be estimated by their ratio to the white cells in the thick film, again assuming that the number of white cells is not subject to extreme variation.

Enumeration of the parasites is most easily done by using the ratio of parasites to the actual number of blood cells. When parasites are numerous their ratio to red blood cells is used; this ratio is obtained from the stained thin smear and the actual number of red cells, determined from blood obtained at the time the smear was made. When parasites are less numerous the ratio of parasites to white cells is determined in the thick film and the white cells counted in blood obtained at the same time. This method gives the number of parasites per cu. mm. of blood, just as blood cells are reported.

Ratio: 4 parasites per white cell
White cell count: 4800 per cu. mm.
Parasite density: 19,200 per cu. mm.
Ratio: 1 parasite per 80 red cells
Red cell count: 4,000,000 per cu. mm.
Parasite density: 50,000 per cu. mm.

It is also possible to count parasites directly, as blood counts are made. If a known volume of blood is placed on a known area of a slide the depth of the blood can be computed. After this blood is laked and stained, parasites can be counted in each microscopic field. By calibrating the microscope the volume of blood covered by each field can be determined.

Autopsy Material. Postmortem examinations may be necessary to establish the diagnosis of infection with *Plasmodium falciparum*. Smears are better than tissue sections for this purpose and should be made from liver, spleen, bone marrow, and gray matter of the brain. Such preparations may be stained with Wright's or fixed with methyl alcohol and stained with Giemsa.

In fixing tissue for section, formalin should be avoided; it produces a pigment which may be confused with that of malaria and causes the parasites to stain poorly. Zenker fixation is recommended for most tissues infected with protozoa.

Zenker's Fluid

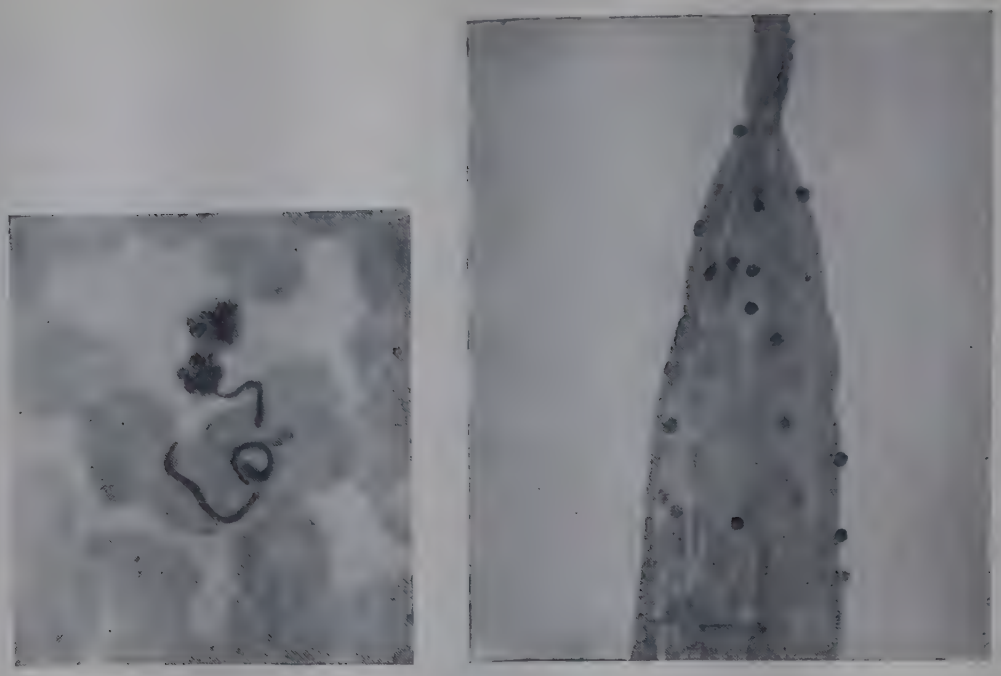
Potassium bichromate	2.5 Gm.
Mercuric chloride	5.0 Gm.
Water	100.0 ml.

Just before using, 5.0 ml. glacial acetic acid are added.

Lendrum (1944) has suggested fixation in an aqueous solution of 2.5 per cent potassium bichromate and 5 per cent mercuric chloride for six hours; the tissue is then transferred to 5 per cent mercuric chloride.

Exflagellation. The change from microgametocytes to microgametes, normally occurring in the mosquito, may be duplicated in the laboratory at room temperature. The blood must be prevented from clotting or drying, best accomplished by placing a heavy thin smear in a Petri dish containing moistened filter paper. Gametes usually develop within 30 minutes; increasing atmospheric carbon dioxide by breathing on the preparation hastens the process. The smears are dried and stained by the usual methods. Accidental distortion of the gametocytes may be found in citrated blood which has been standing for some time at room temperature.

Dissection of Mosquitoes for Malaria Parasites. In selecting mosquitoes for dissection, use of those containing recently ingested blood and those in which the abdomen is distended by fully developed ovaries is avoided. Recently engorged mosquitoes will

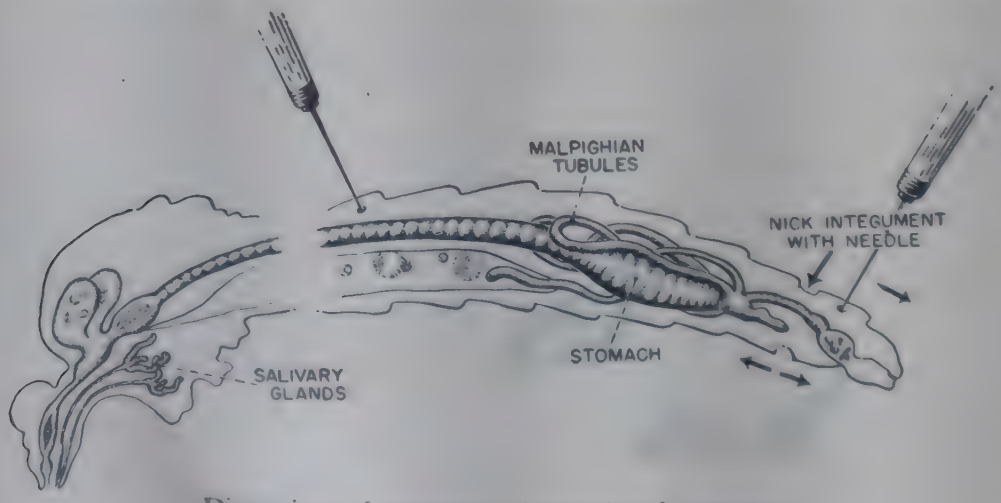


(*Left*) Exflagellating microgametocyte of *Plasmodium vivax.* (*Right*) Oöcysts of *Plasmodium vivax* on stomach of mosquito. (Photomicrographs (*left*) × 1200, (*right*) × 150.)

show a dark, swollen area in the ventral abdominal region; these may be kept in tubes with moist cotton plugs or in humid cages for 48 hours to allow digestion of the blood meal. When it is necessary to examine mosquitoes that have recently fed, the abdomen is carefully pricked and the blood squeezed out.

The mosquito is killed with chloroform and the species identified. Then the legs and wings are removed from the body with sharp dissecting needles. The head and thorax are separated from the abdomen by a clean cut with a razor blade and the former is transferred to a second slide on which a drop of saline tinted with methylene blue has been placed.

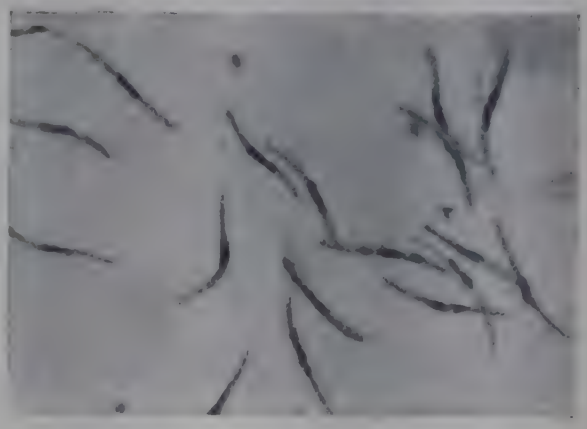
STOMACH. The slide containing the abdomen is placed on a black background and illuminated with strong direct light. A large drop of physiological saline is added and, with needles, the integument is nicked on both sides just in front of the terminal segments. Then one needle is placed on the terminal segments and the other on the integument at the anterior end. By gentle traction, the stomach is drawn out with the attached Malpighian tubules and ovaries. The excess saline is removed with a point of filter paper and the stomach freed from the attached organs. With the microscope, the stomach may then be examined in fresh saline under a coverglass. Oöcysts are found on



Dissection of mosquito for malarial parasites.

the outside of the stomach wall, appearing as protrusions which vary from 6 to 80μ in size. A positive diagnosis should not be made unless pigment granules are visible within the objects, as small protrusions of the stomach wall or fat cells may simulate immature oocysts. It must also be remembered that it is not possible to distinguish between bird and human malaria in the mosquito.

SALIVARY GLANDS. Under a dissecting microscope, the head and thorax are arranged on the slide with the head to the right. The thorax is held with a needle in the left hand and the head is drawn down and forward with the right. Usually the salivary glands will come out in a tag of tissue attached to the head. They may be recognized by their trilobed form, shining appearance, and tint from methylene blue absorbed from the saline; if they are not extracted when the head is removed they may be recovered by tearing apart the tissues near the neck attachment. After isolation, the glands may be mounted under a coverglass. Slight pressure will rupture them and release the sporozoites. Under the high-power lens the sporozoites are slender, spindle-shaped bodies. They may be stained with Giemsa or Wright's stain after allowing the saline preparation to dry; under the oil-immersion lens the blue-stained spindles with a central red chromatin dot are found to be 12 to 14μ long.



Sporozoites of *Plasmodium vivax*. (Photomicrograph × 1200.)

Flagellates of Blood and Tissues

The most important flagellates of man are those that live in the blood and tissues, the hemoflagellates. Diseases caused by these parasites are second only to malaria in incidence and importance in parts of India and China. Certain areas of Africa have been rendered almost uninhabitable by epidemics of sleeping sickness. While only occasionally seen outside the endemic areas, infections caused by these parasites have occurred in some military personnel returning from World War II. These diseases constitute an important part of tropical medicine, and the individual working in parasitology should become familiar with their epidemiology and diagnosis. As in malaria, there is an alternation between a vertebrate and an insect host in the life cycles.

Classification. Parasitic protozoa of the class Mastigophora and belonging to the family Trypanosomidae Doflein, 1901, are primarily parasites of insects, some having become adapted to vertebrates or plants. This family includes six genera, only the two using vertebrates as their definitive hosts being of medical importance:

Leishmania donovani (Laveran and Mesnil, 1903).
Leishmania tropica (Wright, 1903).
Leishmania brasiliensis Vianna, 1911.
Trypanosoma gambiense Dutton, 1902.
Trypanosoma rhodesiense Stephens and Fantham, 1910.
Trypanosoma cruzi Chagas, 1909.

Parasites of the genera *Leptomonas, Crithidia,* and *Herpetomonas* involve only invertebrates; those of the remaining genus, *Phytomonas,* require plants and invertebrates in their life cycle.

Morphology. The forms assumed by the various species differ during their life cycles in the different hosts or under laboratory conditions. Descriptive names are used for these forms and are readily confused with some of the above generic names. The forms or stages are four in number, and it will be noted that all species exist as flagellates at some time.

Table 54 lists the species of the hemoflagellates and the forms which develop in the life cycle of each.

Table 54

MORPHOLOGIC FORMS

	Leishmanial	*Leptomonad*	*Crithidial*	*Trypanosomal*	
				Metacyclic	*Mature*
L. donovani *L. tropica* *L. brasiliensis*	Man	Insect			
T. gambiense *T. rhodesiense*			Insect	Insect	Man
T. cruzi	Man	Man	Man, insect	Insect	Man

LEISHMANIAL FORM. This is the usual intracellular stage found in the vertebrate host infected by *Leishmania* or *Trypanosoma cruzi.* The organism is round or ovoid and has no free flagellum. Well-stained specimens show a nucleus, parabasal body, and blepharoplast.

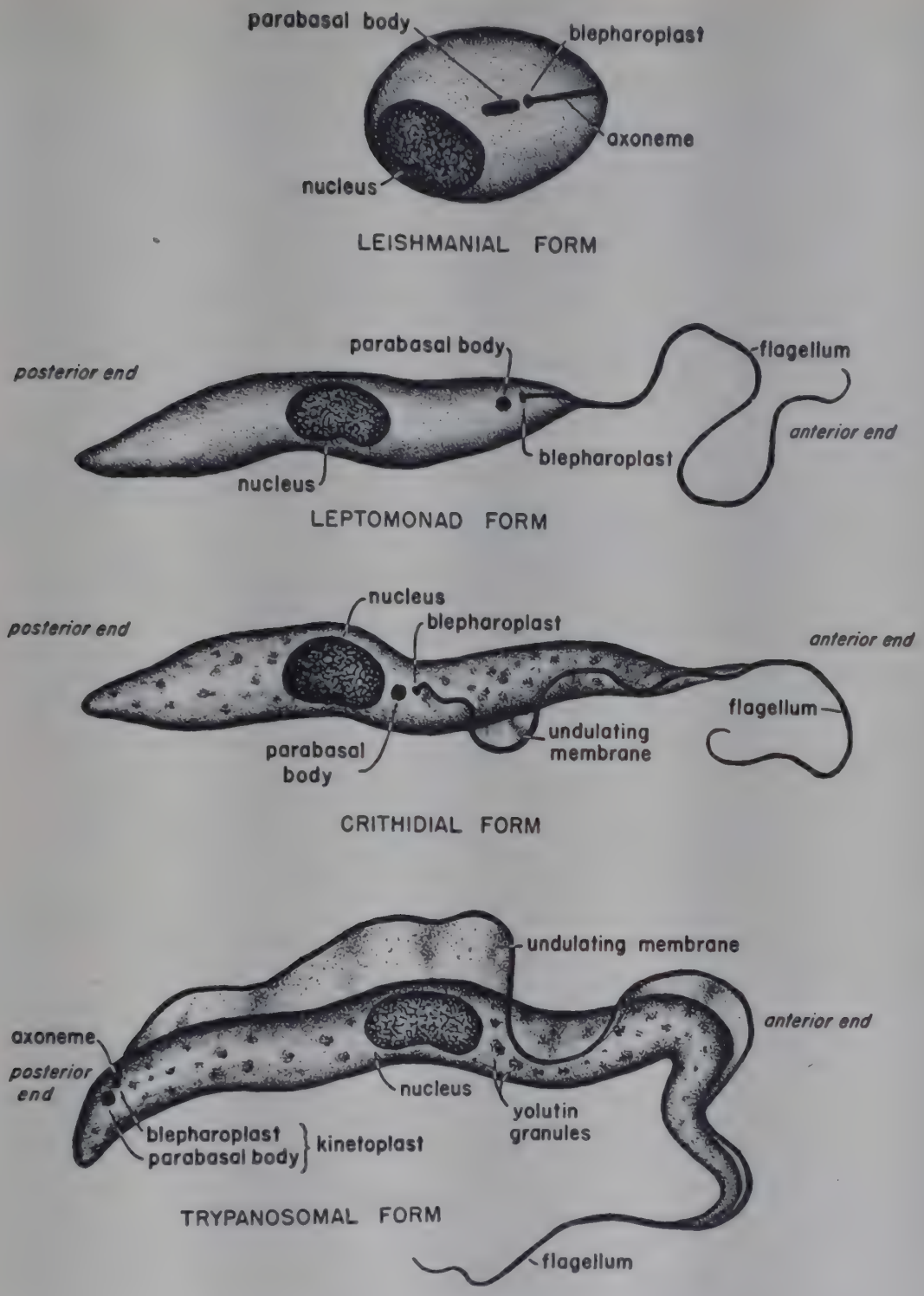
LEPTOMONAD FORM. This stage is found in the insect host and in cultures of parasites of the genus *Leishmania.* It may occur as an intracellular stage of *T. cruzi.* The organism is elongated and flattened with a free flagellum arising from the anterior end.

CRITHIDIAL FORM. Parasites of the genus *Trypanosoma* develop this stage in the invertebrate host and in cultures. The parabasal body and the blepharoplast are just anterior to the nucleus and give rise to a short undulating membrane, with or without a free flagellum.

TRYPANOSOMAL FORM. This stage, found in parasites of the genus *Trypanosoma,* exists in two phases: the metacyclic phase (infective stage) is found in the invertebrate and the mature phase in the vertebrate host. The parabasal body and the blepharoplast are located between the posterior end of the organism and its nucleus, giving rise to a long undulating membrane. Those that vary in shape and size are called *polymorphic.*

LEISHMANIA

Parasites of the genus *Leishmania* Ross, 1903, produce three clinical diseases differentiated by the organs or tissues involved. *Visceral* leishmaniasis or *kala-azar,* caused by *Leishmania donovani,* is an important disease of China and India and



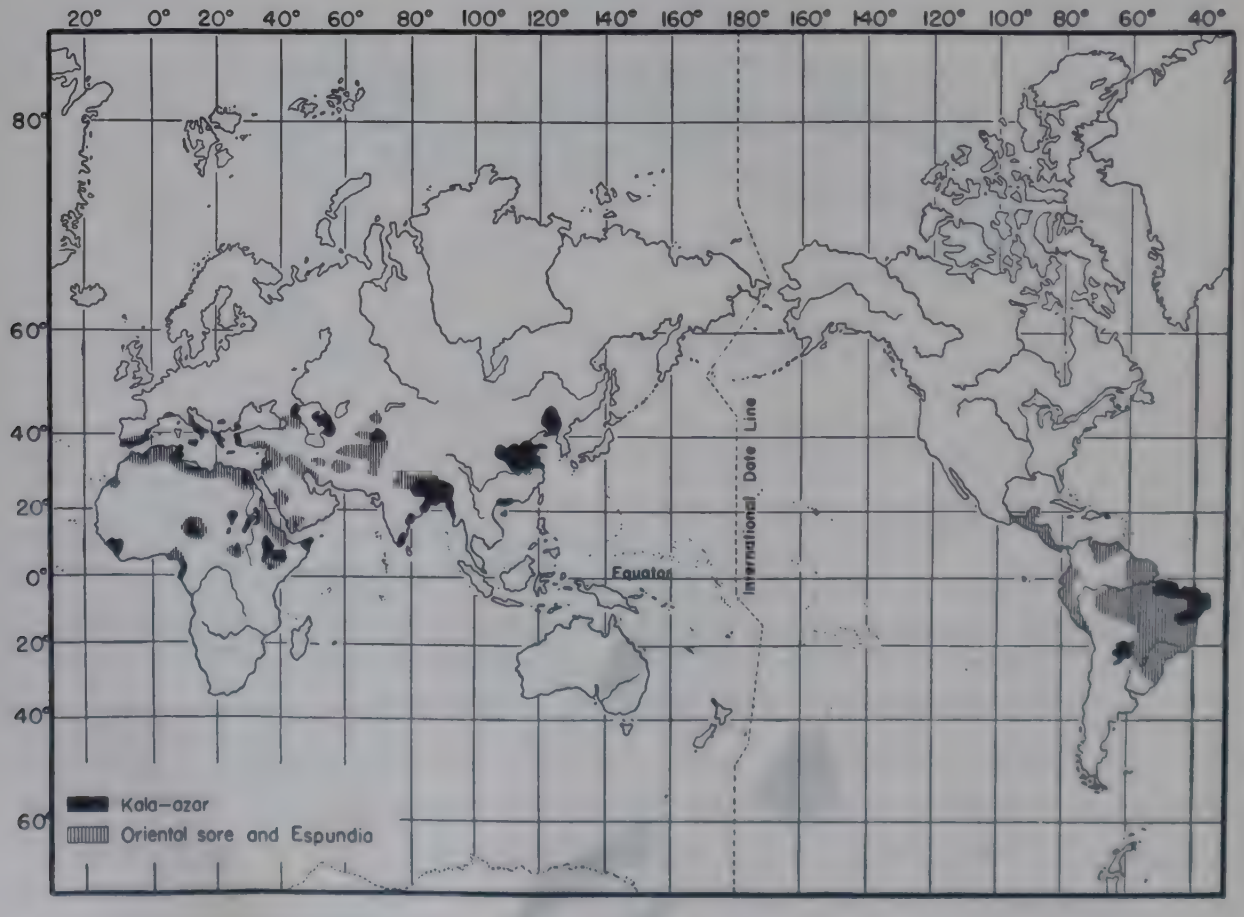
Morphologic forms of the hemoflagellates.

is also found in Africa and South America. Some American servicemen have contracted the disease (Wand and Kruger, 1947). A closely related disease of the Mediterranean region, more common in children, is sometimes ascribed to a separate species, *L. infantum* Nicolle, 1908; most parasitologists consider this designation to be a synonym for *L. donovani*. Two types of *cutaneous* infections occur in separate parts of the world: *oriental sore* or *cutaneous* leishmaniasis, caused by *L. tropica,* is prevalent in the Near East and the Mediterranean region; *espundia* or *mucocutaneous* leishmaniasis, caused by *L. brasiliensis,* is almost exclusively confined to Central and South America; recently an autochthonous case has been reported from Texas (Stewart and Pilcher, 1945).

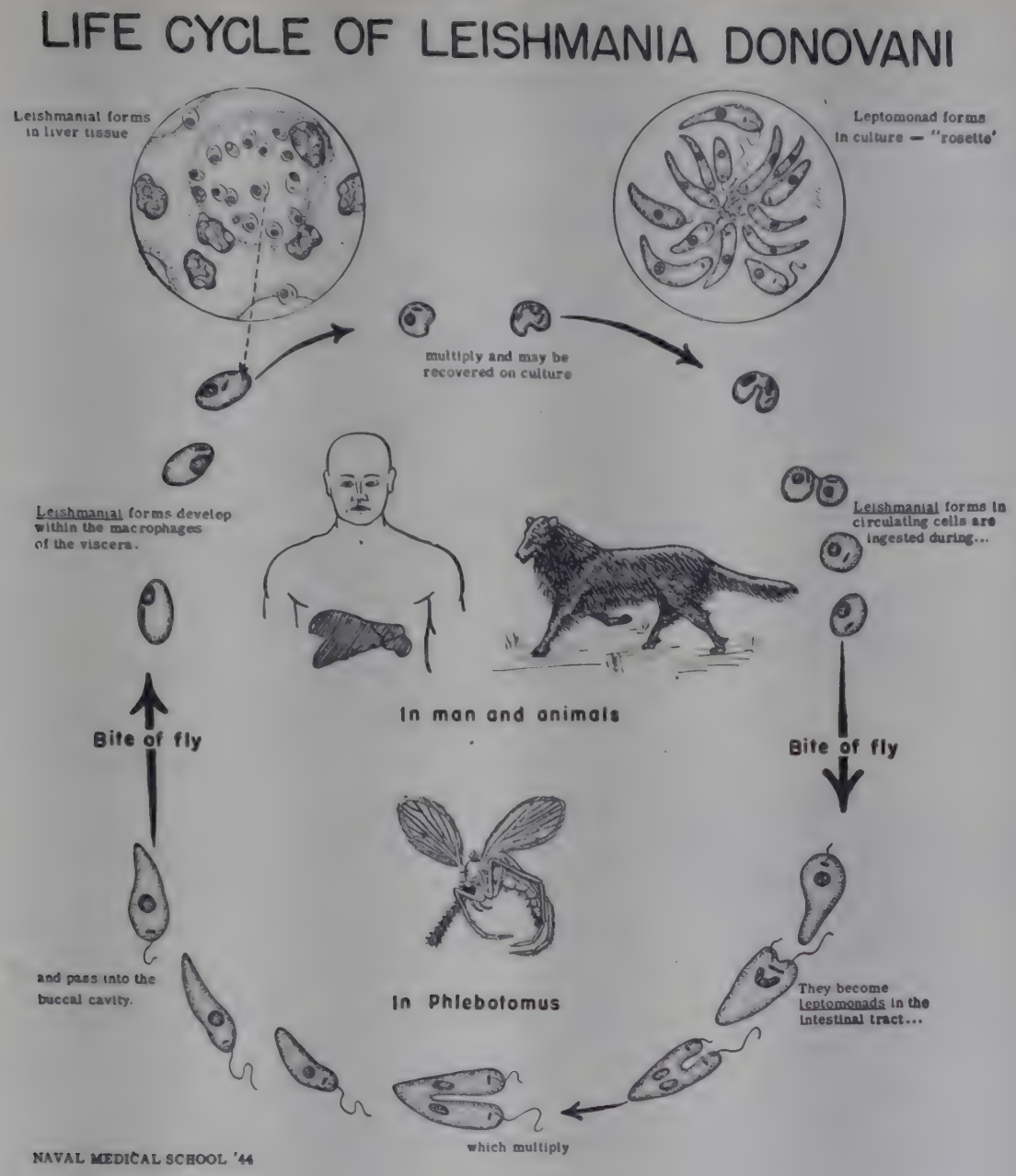
Life Cycle. Available information regarding the life histories of the parasites of this genus indicates that they are much alike. In some areas of the world, kala-azar is associated with a closely related disease of dogs, and oriental sore is frequently encountered as a natural disease in them (Malamos, 1947). Other mammals, especially wild rodents, are implicated as reservoir hosts. The vertebrate is usually accepted to be the definitive host with sandflies of the genus *Phlebotomus* serving as intermediate hosts and vectors. Certain features regarding the transmission by *Phlebotomus* flies in nature still remain to be clarified. The epidemiology of espundia indicates that a flying, biting insect is involved in the transmission of the parasite; a reservoir host other than man is not known.

The leishmanial forms in the mammalian host are ingested by the biting insect. Under laboratory conditions, these develop into leptomonad forms in the gut of the vector, multiply by longitudinal fission, and later return to the insect's pharynx. It is believed that the infective forms are introduced into man when the fly again feeds. The parasites lodge in macrophages (reticulo-endothelial cells) of the definitive host where they multiply by binary fission of the leishmanial forms. When crowded with parasites, the cells rupture and free the leishmania to invade new cells. Circulating parasites and those in skin lesions are available to the feeding insect. Since the etiologic agent of kala-azar may be recovered from the upper respiratory tract and from the feces, direct contagion must be considered as a possible means of spread. Cutaneous lesions may also be infectious.

Prophylaxis. Prevention of leishmaniasis is accomplished by control of the



Geographic distribution of the leishmaniases.



Life cycle of *Leishmania donovani.*

insect vector (see Medical Entomology). Dogs known to harbor the parasites should be destroyed since they are very resistant to treatment (Adler and Tchernomoritz, 1946). Human cases with infectious cutaneous lesions should be isolated and all infections treated. In hyperendemic areas inoculations of cultures of *L. tropica* have proved to be valuable in preventing disfiguration from oriental sore (Ansari, 1946).

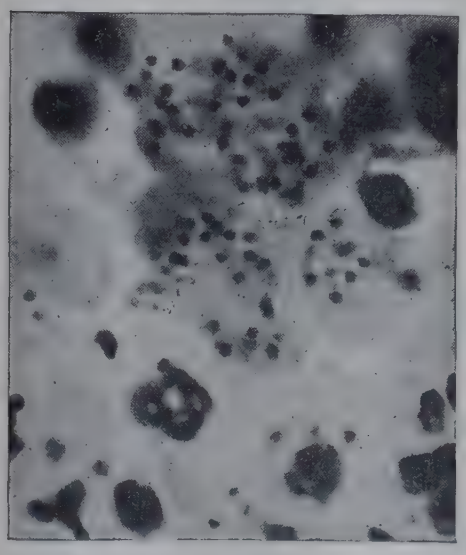
Clinical Illness: KALA-AZAR. The incubation period is variable, usually considered to be a few months. The onset is gradual or sudden and the disease runs an acute, subacute, or chronic course. Characteristic features include an irregular, recurring fever, leukopenia, and enlargement of liver and spleen, any of which may be confused with symptoms of malaria. The parasites may invade the intestine, resulting in ulcers, secondary infection, and dysentery. The disease may become recurrent or chronic with persistent fever, emaciation, pigmentation of the skin, and intercurrent infection resulting in death. Post-kala-azar skin lesions may develop.

Oriental Sore. This infection begins with a small, indurated papule, usually located on an exposed surface of the body. Lesions may be single or multiple, becoming ulcerated and crusted. Healing usually occurs within a year and leaves a depressed scar.

Espundia or Mucocutaneous Leishmaniasis (American Leishmaniasis, Uta, Forest Yaws). This disease may involve the skin alone or the skin and mucous membranes. The initial lesion appears on an exposed surface—face, arms, or legs. The ulceration that develops is more extensive than that seen in oriental sore, but eventually heals. About 20 per cent of the patients show subsequent involvement of the mouth, nose, or throat, suggesting hematogenous spread of the organisms. Extensive ulceration and mutilation develop in these patients.

Animals that have recovered from an infection with *Leishmania donovani* have an immunity to reinfection with the same organism or infection with *L. tropica.* On the other hand, infection with *L. tropica* produces an immunity to reinfection with only the same species.

Diagnosis. A diagnosis of leishmaniasis is made from material obtained from the appropriate site. Such material may be examined by staining or culturing or by injection into animals with subsequent autopsy. Parasites are occasionally found in the blood of patients with kala-azar; more commonly it is necessary to aspirate liver, spleen, or bone marrow in order to find them. Shortt (1947) has given an excellent critical summary of the methods of diagnosis for kala-azar. In cutaneous leishmaniasis the parasites are found in cells or fluid obtained from the base or perimeter of the ulceration. The margins or base may be scraped with a scalpel or the indurated border aspirated with needle and syringe.



Leishmanial forms of *Leishmania tropica* in skin. (Photomicrograph × 1200.)

It must be emphasized that these parasites cannot be distinguished from each other on the basis of morphologic characters. They are identified as Leishman-Donovan bodies in the direct stained smear. These are to be found within macrophage cells or the neutrophils and monocytes of blood smears. The individual parasite is ovoid, 2 or 3μ in diameter. The nucleus, parabasal body, and blepharoplast are visualized with the aid of the polychrome stains; the cytoplasm is light blue and the chromatin material red to purple.

Leptomonad forms are obtained by culturing infected blood or material obtained by aspiration. These appear as flagellated forms without an undulating membrane; they are often arranged in a rosette pattern. With appropriate stains, they show blue cytoplasm, a red nucleus, and a darker-staining parabasal body.

Additional presumptive tests which depend upon biochemic changes in the blood may also be used. In kala-azar, the formol-gel or aldehyde test is the one

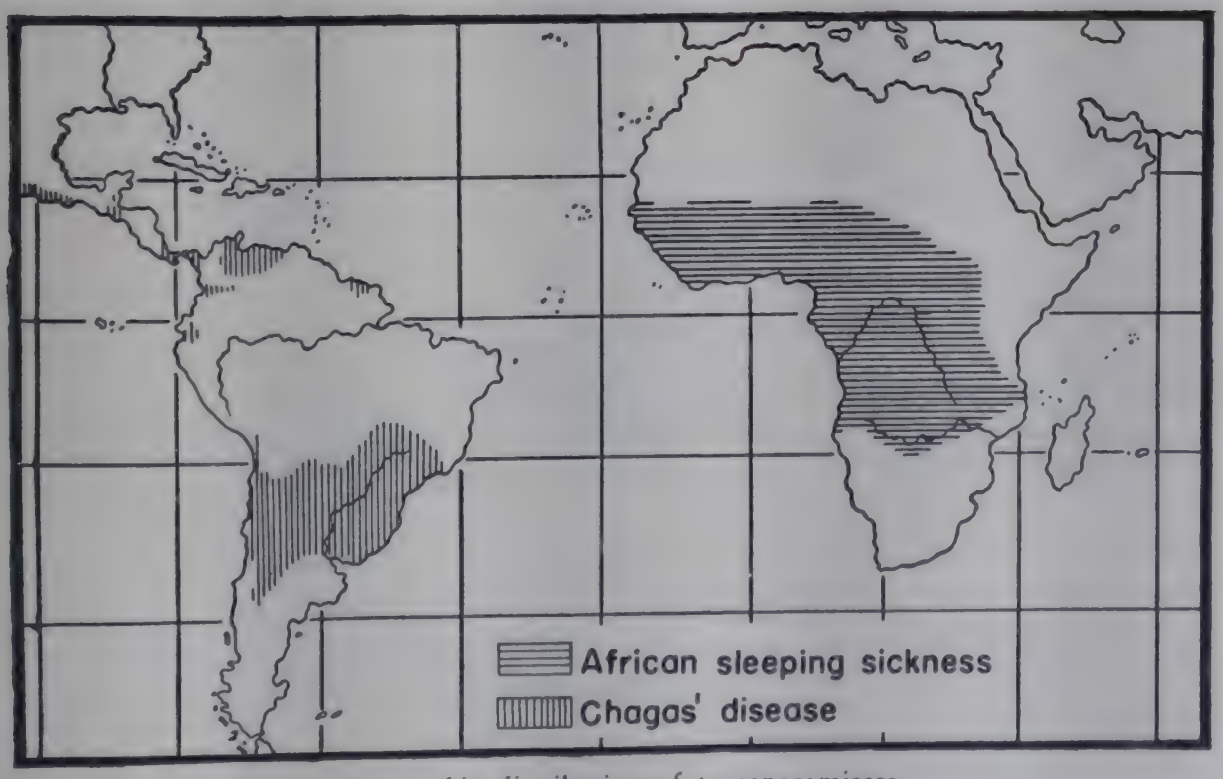
most commonly employed; complement-fixation has recently given very promising results (Sen Gupta, 1944; Ghosh et al., 1945). Dostrovsky and Sager (1946) have highly recommended an intracutaneous test with leishmania vaccine in the diagnosis of oriental sore. The Montenegro skin test is of value in diagnosing mucocutaneous leishmaniasis (Lopez and Leander, 1945; Cerruti, 1945; Iriarte, 1946).

Trypanosomes

The hemoflagellates of the genus *Trypanosoma* Gruby, 1843, are divided into many species, three of which may infect man. These species of medical importance are limited in their geographic distribution and are divided into two groups which differ in the forms encountered in their life cycles, in their vectors, and in other aspects of epidemiology. African trypanosomiasis, or sleeping sickness, is caused by two species, *Trypanosoma gambiense* of West Africa and *T. rhodesiense* of East Africa. An interesting discussion of the relationship of these species to the closely related *T. brucei* is given by Fairbairn and Burtt (1946). American trypanosomiasis, or Chagas' disease, caused by *T. cruzi,* is found in parts of Mexico and Central and South America.

In addition to those listed above, several species are found in other vertebrates; a few of these are listed:

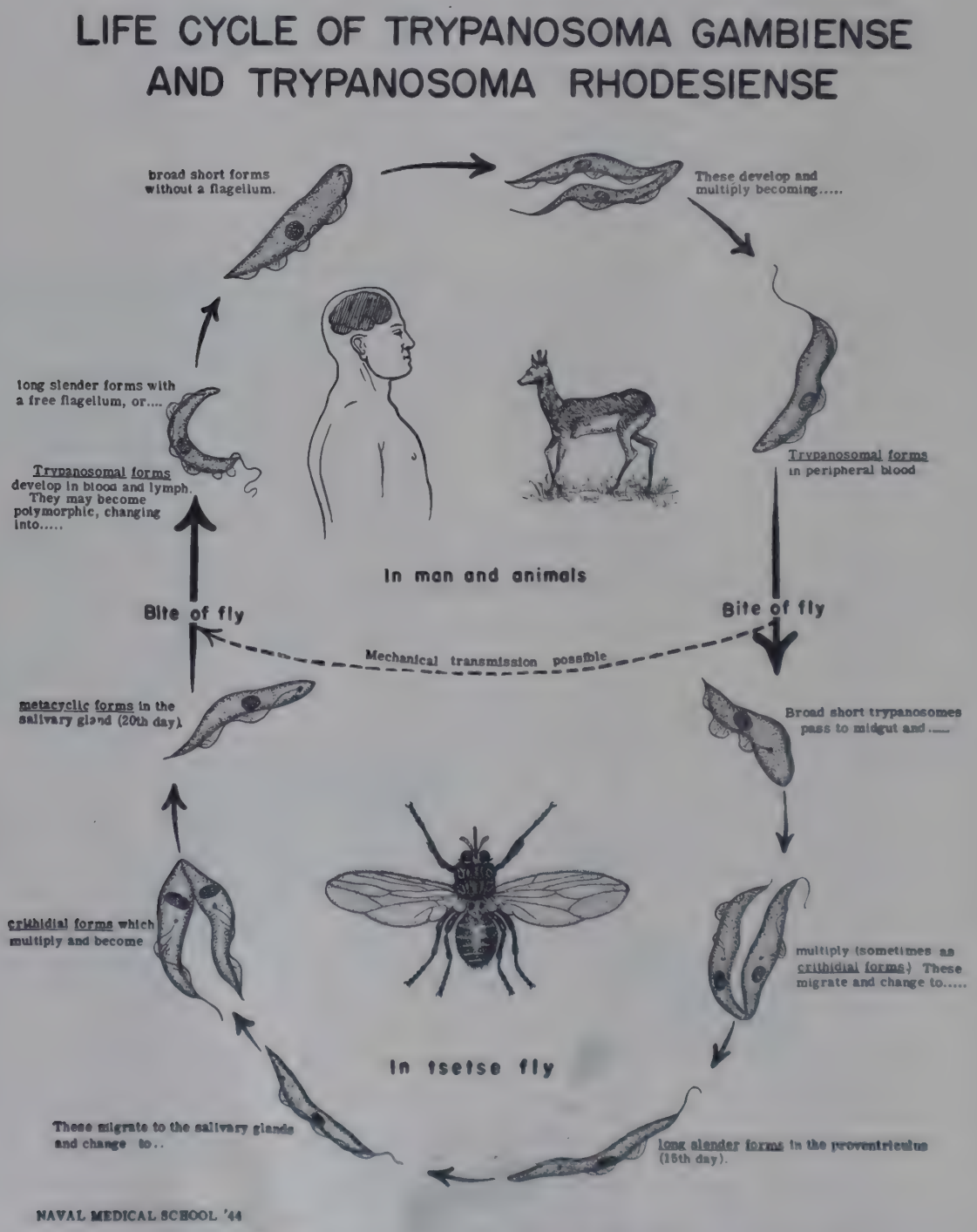
Species	*Definitive Hosts*	*Vector*	*Distribution*	*Disease*
T. brucei	Domestic animals, wild game	Tsetse flies	Africa	Nagana
T. lewisi	Rats	Fleas	Cosmopolitan	None
T. equiperdum	Horse, ass	None (by coitus)	Cosmopolitan	Dourine
T. evansi	Domestic animals	Horseflies	Cosmopolitan	Surra



Geographic distribution of trypanosomiases.

African Trypanosomiasis: LIFE CYCLE. Man is an important reservoir of infection, wild animals, especially the antelope, also serving in this capacity. Trypanosomes which are circulating in the blood of the definitive host are picked up by the biting tsetse fly (*Glossina* species). The parasites undergo development and multiplication within the insect vector. At the end of about 20 days, development is completed and organisms are present in the salivary glands—an *anterior station* parasite. Metacyclic forms are injected when the fly feeds again, and the insect usually remains infective for life. Mechanical transmission may occur when a fly whose proboscis has been contaminated by trypanosomes feeds again within a short time.

The metacyclic forms become mature trypanosomes and multiply within the



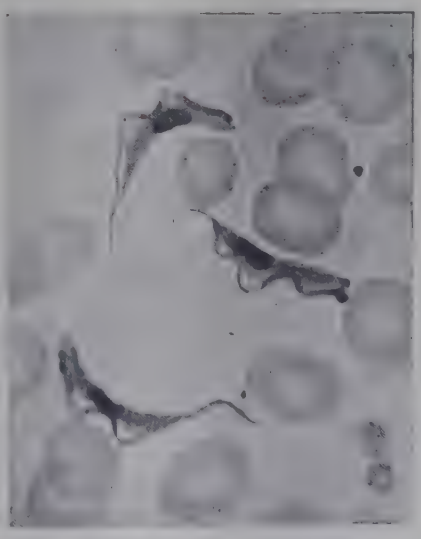
Life cycle of *Trypanosoma gambiense* and *Trypanosoma rhodesiense*.

blood and lymph systems of the mammalian host. Multiplication is by longitudinal fission, only the trypanosomal form being present.

Prophylaxis. Protection from the bites of tsetse flies and control of these insects (see p. 701) will prevent infection. Chemoprophylaxis is also available (1.0 Gm. of Bayer 205 every two or three months). The reservoir of infection may be reduced by adequate therapy of all cases occurring in human beings; eradication of the animal reservoir has been used in control of *Trypanosoma rhodesiense*.

Clinical Illness. African trypanosomiasis is an acute febrile illness which may last for months or years, with or without remissions. The bite of the infected fly may produce a local inflammatory reaction which persists for two or three days. After an incubation period of 10 days to three weeks, sometimes several months, a lymphadenitis develops, frequently involving the posterior cervical glands (Winterbottom's sign). Peculiar skin rashes may develop in white individuals who are considered to be more susceptible to the disease than natives. Local areas of edema are frequently seen with transitory involvement of hands, feet, orbital region, or joints. This period of invasion is followed by a second stage which affects the central nervous system, from which the disease derives its name of sleeping sickness. The early manifestations may be mild or acute, progressing to marked somnolence and emaciation due to starvation. When this stage has been reached the prognosis is poor. *Trypanosoma rhodesiense* causes a more acute illness which may result in death within a year.

Diagnosis. The trypanosomes may be recovered from lymph nodes, blood, or spinal fluid. Early in the disease, best results are obtained by examination of material obtained from an enlarged lymph node. Later the parasites may be readily recovered from the blood and occasionally from the spinal fluid. These polymorphic forms range from 15 to 30μ in length and are not within blood or tissue cells. In fresh blood the organisms are detected by their active movement which disturbs the red blood cells. The stained preparations show the parasite in its flagellated form with an undulating membrane usually visible. The cytoplasm stains blue with the chromatin red or dark red. Cultures and animal inoculation may be employed when direct examination fails to confirm the diagnosis. However, cultures of *Trypanosoma gambiense* and *T. rhodesiense* are less satisfactory than with the other hemoflagellates. Fain (1944) believes sternal puncture to be of some value.



Trypanosomal forms of *Trypanosoma gambiense* in peripheral blood. (Photomicrograph × 1200.)

American Trypanosomiasis: Life Cycle. Man and other mammals, especially the armadillo and opossum, constitute a reservoir of infection. Reduviid bugs (cone-nose, "assassin," or "kissing" bugs) serve as the intermediate host and vector. The trypanosomal forms of *Trypanosoma cruzi* are ingested by the insect as it feeds. These multiply in the gut of the insect as crithid-

ial forms and are found in the feces as metacyclic trypanosomes in about 10 days; the bug remains infective for life. When the vector feeds, it deposits feces containing the infective stage of the parasite; the organism is called a *posterior station parasite* in contrast to those that are transmitted in the salivary fluid and by the mouth parts (anterior station).

Man becomes infected when the parasites in the feces are rubbed into the abraded skin or the wound produced by the bite. The trypanosomes invade tissue cells, assume the leishmanial form, and multiply by binary fission; dividing trypanosomal forms are not found in the blood. The infected cells are destroyed and the parasites are liberated to invade new cells or to become trypanosomal forms which circulate in the blood.

Prophylaxis. Prevention of infection is accomplished by avoiding the insect vector. Although infected bugs are found in the United States, clinical infections have not been seen outside the endemic areas.

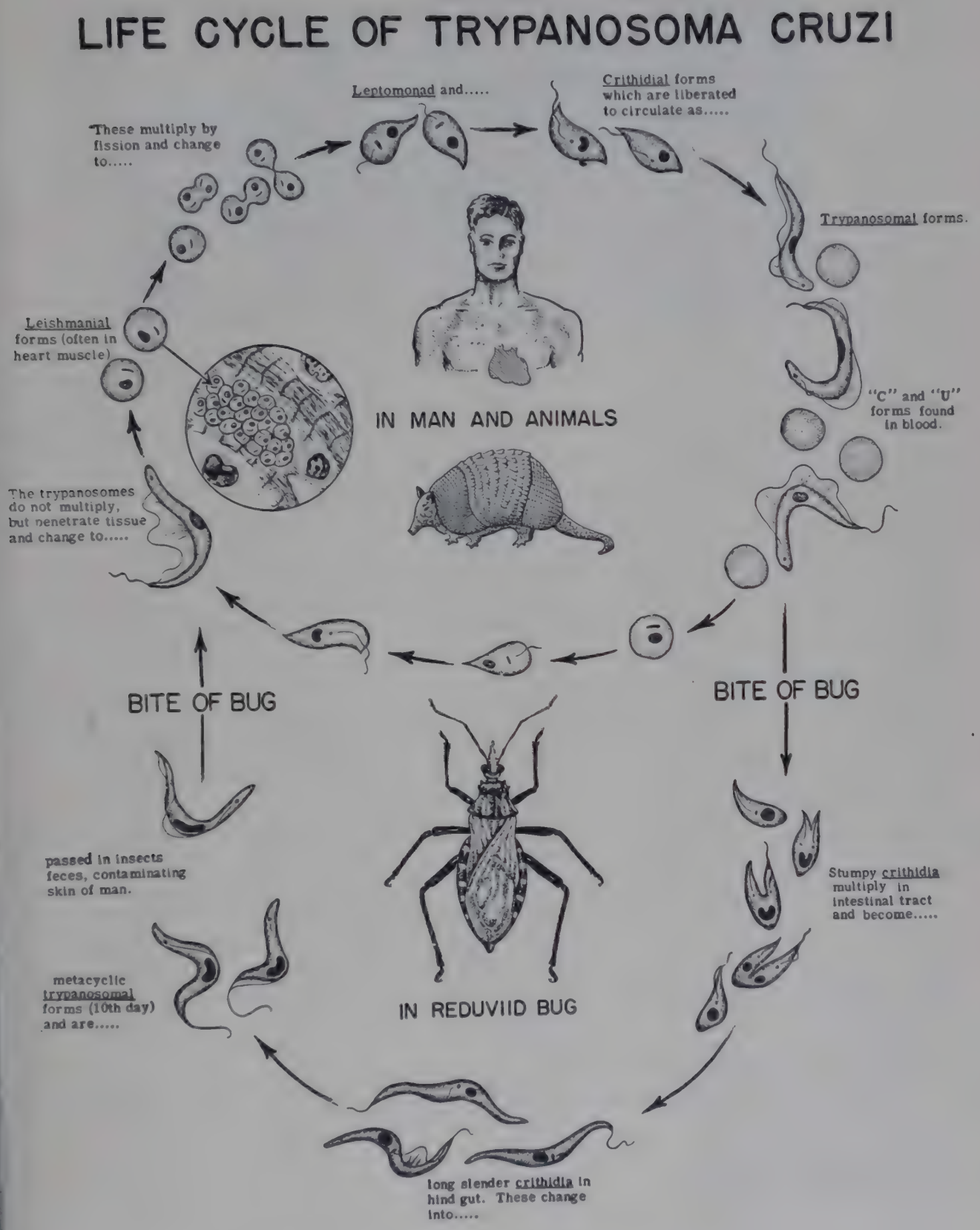
Clinical Illness. Chagas' disease is seen most frequently in children, and in the young individual the disease has a more acute form. After an incubation period of one to two weeks, a sustained high fever develops. There is often a unilateral conjunctivitis or edema of the face suggesting the site of inoculation. Trypanosomes are usually demonstrable during the febrile stage, disappearing when the temperature recedes. The liver and spleen may be enlarged as well as superficial lymph nodes. This acute stage of infection is usually of short duration, often terminating fatally in infants and young children. It may be followed by a chronic form of the disease in which circulating parasites and fever are absent. Symptoms are related to the tissues invaded and may be unimportant; involvement of cardiac muscle may cause sequelae or death. The use of *T. cruzi* against cancer (Roskin, 1946) is an interesting development although it is probably of very little significance.

Diagnosis. A diagnosis of American trypanosomiasis may be confirmed by demonstrating the trypanosomal forms in the blood or the leishmanial forms in tissue. The former are actively motile in fresh blood smears; they average 20μ in length and are characteristically C- or U-shaped when stained. Dividing forms are absent.

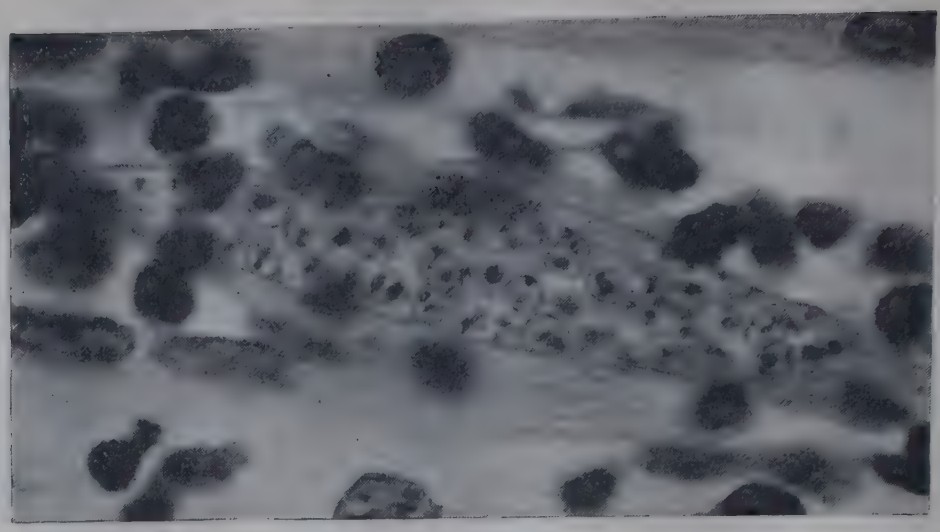
The leishmanial forms may be found in stained sections of many tissues, the heart being frequently involved. They are found in colonies or nests which crowd the surrounding tissue cells or fibers. With appropriate stains the characters are the same as for *Leishmania*. The organisms are readily cultured, and most laboratory animals are susceptible. A diagnosis may also be established by allowing a noninfected reduviid to feed on persons suspected of having the disease—xenodiagnosis. A complement-fixation test has been reported as satisfactory by some workers.

Laboratory Technics for Detection of Flagellates of Blood and Tissues

Fresh Blood Smears. Only the trypanosomes may be detected by this method. A very small drop of blood is placed on the glass slide and spread by placing a coverslip on it.



Life cycle of *Trypanosoma cruzi.*



Nest of leishmanial forms of *Trypanosoma cruzi* in heart muscle. (Photomicrograph × 1200.)

Movement of the parasite causes a characteristic movement of the surrounding blood cells. The examination will fail if too much blood is used.

Stained Blood Smears. Thick or thin smears, stained as for malaria parasites, are satisfactory for trypanosomes. Leishmanial forms may be destroyed by aqueous stains and special fixation is required for them in thick blood smears. The dried thick film is flooded with a solution of acetic and tartaric acids (4 parts of 2.5 per cent aqueous solution of glacial acetic acid and 1 part of 2 per cent aqueous solution of tartaric acid). A grayish-white color indicates that dehemoglobinization of the blood film is complete. Then the film is fixed with methyl alcohol and stained with Giemsa or similar stains.

Blood Concentration (for Trypanosomes Only). Blood is collected in 1 per cent citrate-physiological sodium chloride solution. After filtering through cheesecloth, it is centrifuged for 20 minutes. The cells are separated and hemolyzed by adding 2 per cent acetic acid or distilled water. After centrifuging again for 30 minutes, smears are made of the sediment and stained for parasites.

Aspiration and Biopsy. When searching for leishmanial forms, dry needles and syringes should be used. A small amount of saline may facilitate the aspiration of lymph nodes for trypanosomes. These procedures require surgical asepsis, a local anesthetic, and a careful technic suitable for the tissue being examined. Spinal fluid should be centrifuged and the sediment examined for motile organisms and by staining and culture. Lesions of the skin or mucous membrane may be scraped until bleeding occurs. After hemorrhage has stopped the blood is removed and the serous exudate obtained for examination. Biopsies may also be taken and stained sections and smears examined as for autopsy material in malaria.

Inoculation of Animals. Suspected material may be injected into laboratory animals by subcutaneous, intramuscular, intraperitoneal, or intravenous routes. The following animals are most commonly used:

T. gambiense, *T. rhodesiense*, *T. cruzi* ..... Rats, mice, guinea-pigs, dogs, cats

L. donovani ..... Hamsters

At the end of the incubation period, blood is examined repeatedly for circulating organisms in trypanosomiasis; autopsy material is examined in sections and smears for the leishmanial forms.

Xenodiagnosis. This method is used only for *Trypanosoma cruzi* infections. Laboratory-reared *Triatoma*, free of parasites, are allowed to feed on the patient. After 10 days they

are examined for metacyclic trypanosomes and the feces injected into rats or mice for confirmation.

Cultures. The NNN (Novy, McNeal, Nicolle) medium has been used for many years for the culture of *Leishmania* and *Trypanosoma cruzi*. It has been found that the method of Senekjie (1939) given below is probably better. The procedure includes two media, the second being used only when contaminating organisms are present.

Blood Culture Medium. Fifty parts of Bacto beef extract are dissolved in 1000 parts distilled water; the solution is heated at 50° C. for one hour, then at 80° C. for five minutes. After filtering, neopeptone, 20 parts, agar (Nobel), 20 parts, and sodium chloride (C.P.), 5 parts, are added. The final pH is adjusted to 7.2 to 7.4, and the mixture autoclaved at 15 pounds for 20 minutes. When cooled to 45° to 50° C., defibrinated rabbit's blood, 10 per cent of the volume, is added. The National Institute of Health has modified this by overlaying the slants with Locke's solution.

Egg-liver Extract. Four eggs are emulsified with glass beads in a sterile flask. To the eggs are added 50 ml. of a solution of sodium chloride, calcium chloride, potassium chloride, and sodium bicarbonate, 0.02 Gm. each, in 100 ml. distilled water. The mixture is emulsified, filtered, made into slants, and sterilized at 10 pounds for 10 minutes. The slant is overlayed with sterile 0.5 per cent liver extract in physiological salt solution. Contaminating organisms are removed by alternate transfers from blood to egg-liver media. The cultures of these parasites grow best at 22° C.

For Trypanosoma Gambiense and T. Rhodesiense (Brutsaert and Henrard, 1937). Five ml. of patient's blood are mixed with 1.0 ml. of 1 per cent sodium polyethanol sulfonate (Roche) or with 1.0 ml. of 0.025 per cent solution of heparin. This is divided into 10 tubes, each containing 2.0 ml. citrated human or guinea-pig blood. It is incubated at 24° to 30° C. and examined after 10 and 20 days by making smears from the surface and depths of the cells. An additional method for the culture of trypanosomes is that of Weinman (1944); it is much more complicated and does not give improved results.

Serologic Methods: Aldehyde Test (Napier) for Leishmania Donovani. One drop of 40 per cent formaldehyde is added to 1.0 ml. of patient's blood serum in a test tube; this mixture is shaken well and allowed to stand at room temperature. If the reaction is positive, the serum immediately becomes opaque and gels within 3 to 30 minutes. If negative, no reaction should occur in 24 hours. Infection of less than four months' duration may produce an opaque serum which does not gel; such doubtful reactions should be confirmed by other tests. A finger-prick test is also available.

Antimony Test (Chopra) for Leishmania Donovani. In a small test tube is placed 0.2 ml. whole serum and in another tube of the same size an equal amount diluted 1 : 10 with distilled water. The contents of each tube are carefully overlayed with a 4 per cent solution of pentavalent antimony (ureastibamine). If the reaction is positive a thick flocculent disc forms at the junction of the two fluids. Reactions which are delayed for a few minutes to an hour suggest an early infection. The diluted serum may give a negative reaction in early cases.

Sia's Test This precipitation test is suggestive evidence of kala-azar. In a small test tube containing 0.6 ml. distilled water are placed 20 cu. mm. blood. The contents of the tube are agitated until mixed. An immediate clouding of the distilled water indicates a positive reaction. The time required for sedimentation—15 minutes to one hour—indicates the strength of the reaction.

Complement-fixation Tests. Such tests appear to be more accurate in the diagnosis of *Trypanosoma cruzi* than in other species. An antigen prepared from cultures of this organism is reported as better than one obtained from infected tissues.

CHAPTER 19

The Intestinal Protozoa

All four classes of protozoa contain species which may be found in the human intestinal tract. These will be discussed in the following pages as the amebae, the flagellates, the ciliates, and the sporozoans. The incidence of these parasites is reflected in the report of Sapero and Johnson (1939) in which 47 per cent of 1021 Navy men were found infected with one or more species of the intestinal protozoa. *Endamoeba coli* and *Endolimax nana* are the species most commonly present; *Endamoeba histolytica* is found in about 10 per cent of those examined in the United States.

The Amebae

The amebae which inhabit the intestinal tract of man are cosmopolitan in their distribution. Only one species possesses proved pathogenicity, and *amebiasis*, the disease caused by this parasite, is one of the more important protozoan infections of man. It may be encountered in any region and epidemics have occurred in the United States; but it is in areas where sanitary conditions are poor that the infection reaches its greatest prevalence. Amebic dysentery is a common disease in the Tropics and the Orient.

Classification. The class Sarcodina includes unicellular animals which are free-living forms, commensal organisms, or true parasites. These protozoans move by means of pseudopodia; of particular interest is the order *Amoebida* Calkins, 1902, in which the pseudopodia do not anastomose. Five species may be encountered in the intestinal tract of man, two belonging to the same genus, *Endamoeba* Leidy, 1879.

Endamoeba histolytica (Schaudinn, 1903) Hickson, 1909.
Endamoeba coli (Grassi, 1879) Hickson, 1909.
Endolimax nana (Wenyon and O'Connor, 1917) Brug, 1918.
Iodamoeba bütschlii (v. Prowazek, 1911) Dobell, 1919.
Dientamoeba fragilis Jepps and Dobell, 1918.

Various strains or races of *Endamoeba histolytica* have been studied for differences in virulence. Large and small races are identified morphologically, the latter appearing to be less pathogenic in experimental animals and man. Small races of *E. coli* are also occasionally encountered.

The nomenclature of the intestinal amebae has been a subject of much controversy. European authors appear to favor the generic name *Entamoeba* Casa-

grandi and Barbagallo, 1895, to *Endamoeba*. Since the International Commission on Zoological Nomenclature has approved the latter, it has been used here pending further action by the Commission. *Iodamoeba williamsi* is a common synonym for *I. bütschlii* and is preferred by some; of the two organisms described and given these two names by v. Prowazek in 1911 and 1912, his description and the specific name *bütschlii* have been accepted. *Dientamoeba fragilis* has been considered by some to be more closely related to the flagellates than to the amebae.

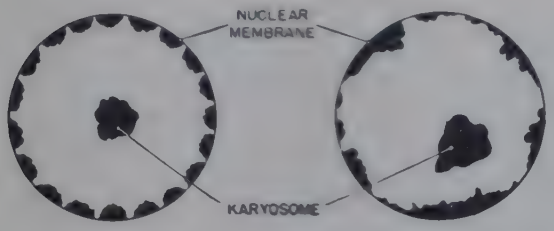


Schematic three-dimensional representation of a cyst of *Endamoeba histolytica.*

Morphology. With one exception, the intestinal amebae exist in two stages: an active or vegetative stage, commonly called the *trophozoite,* and an inactive or resistant stage called the *cyst.* An intermediate or precystic stage is utilized in the description of many authors. As trophozoites, the amebae are capable of moving, feeding, and reproducing; the pseudopodia formed and the resulting motility are often characteristic of the species. Usually a single nucleus is present, two being found only in *D. fragilis* and dividing trophozoites. Ingested food particles, bacteria, and red cells may be contained in food vacuoles visible within the endoplasm. Reproduction is by binary fission. Under certain conditions the trophozoites become *encysted:* The animal rounds up, extrudes undigested food material, becomes smaller, and loses its motility to form the *precystic stage.* A cyst wall is then secreted and food is stored in *glycogen vacuoles* or as chromatoid matter in bars, splinters, or granules. As the cyst matures the food material is ab-

sorbed. Nuclear division may continue after the cyst is formed, resulting in from one to eight nuclei in the mature cysts of the various species. No cysts have been recognized for *D. fragilis*.

There may be difficulty at first in visualizing the protozoan cysts as spherical bodies, although they are seen rolling across the field in wet preparations. The optical section produced by the microscope reveals only a horizontal plane through the cyst; the entire structure is seen by changing focus. In the preceding diagram of a cyst of *Endamoeba histolytica* it will be noted that the nuclei as well as the cyst are spherical. The chromatoid matter is in round bars and the glycogen vacuole is globular.



Characteristic nuclei of genus *Endamoeba*.

The four genera of the amebae may be differentiated by the structure of the nuclei. While these may be visualized in the living state in some species, staining is required to bring out the finer details. The *Endamoeba* nucleus has a relatively small karyosome and a layer of chromatin granules on the inner surface of the nuclear membrane. In optical section it resembles a cart wheel, the karyosome forming the hub and the nuclear membrane with its chromatin granules the rim. Sometimes lines of fine granules radiate from the karyosome to the periphery like spokes of a wheel. The nuclei of the species of *Endamoeba* are quite similar; only when they are faultlessly stained before degenerative changes have occurred can they be differentiated with certainty. The size and position of the karyosome and the deposition of the chromatin granules on the nuclear membrane are the important differential points.


Characteristic nucleus of genus *Endolimax*.

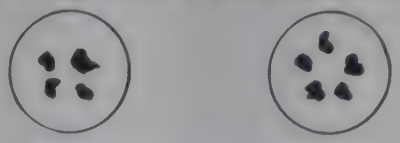
The *Endolimax* nucleus has a relatively large karyosome and no chromatin granules on the nuclear membrane. In optical section it resembles the eye, the karyosome being the iris and the clear space between it and the nuclear membrane the sclera. The nucleus, although essentially the same in both trophozoite and cyst, is much smaller and appears dotlike in the encysted form.



Characteristic nuclei of genus *Iodamoeba*.

The nucleus of *Iodamoeba* is much like that of *Endolimax*, but the karyosome is usually larger and a layer of small chromatin granules lies between it and the nuclear membrane. The karyosome in the cyst is often shifted to an eccentric position, lying against the nuclear membrane to produce a basket-like appearance.

The *Dientamoeba* nucleus has a karyosome made up of granules arranged in a circular or tetrad pattern. As in *Endolimax* and *Iodamoeba,* there are no granules on the membrane.



Characteristic nuclei of genus *Dientamoeba.*

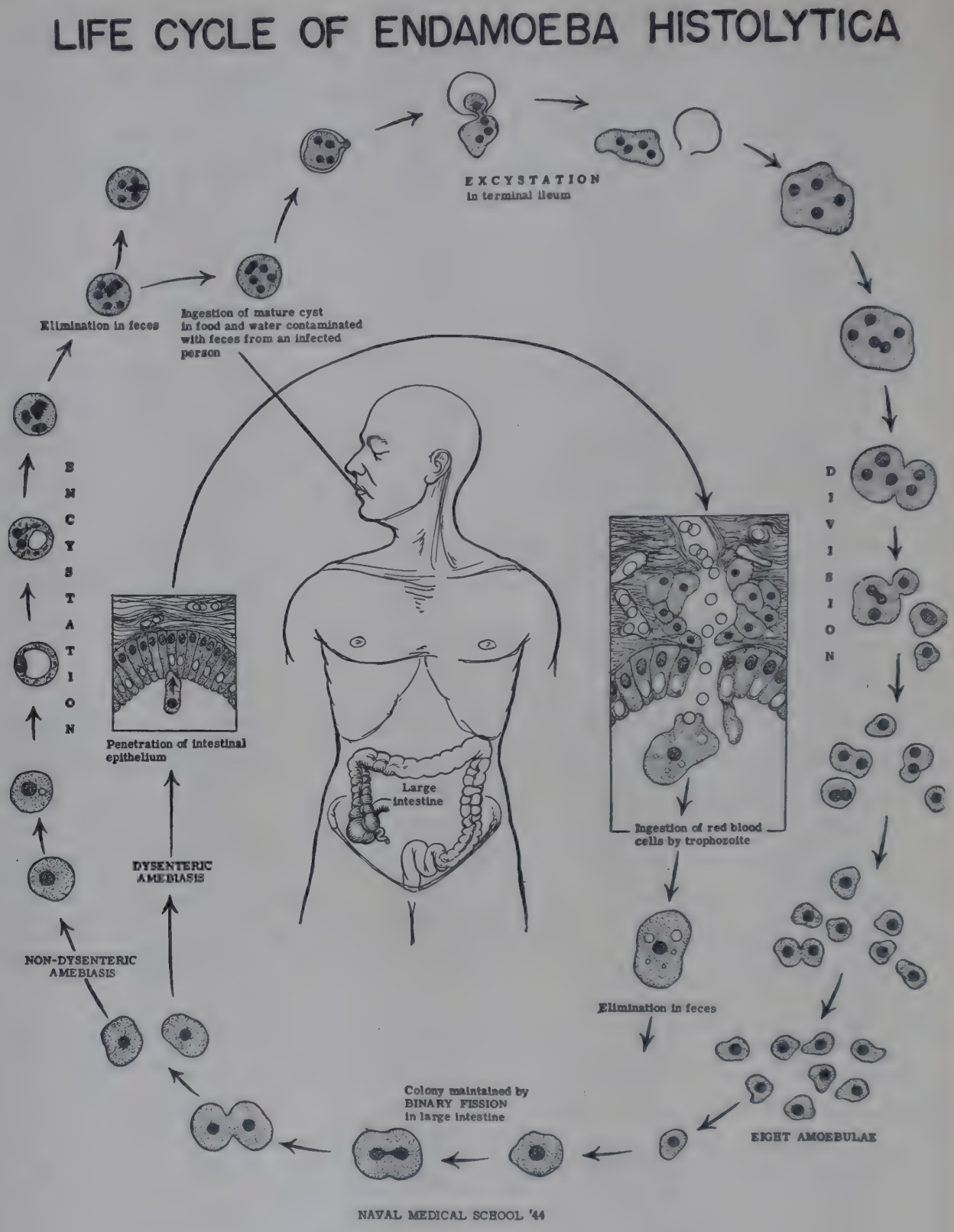
Life Cycles and Transmission. The life histories of the amebae are very simple when compared with those of parasites previously discussed. Man is the principal reservoir host; parasites identical with or closely related to *Endamoeba histolytica* have been demonstrated in naturally infected monkeys, rats, dogs, and pigs. No intermediate host is required, but insects, especially the filth flies, may serve as mechanical vectors.

The preferred habitat of the intestinal amebae is the colon. Here they usually live in the lumen of the bowel, causing the host no annoyance. *Endamoeba histolytica* may invade the bowel wall and produce ulceration and dysentery; the invading trophozoites may be carried to other parts of the body by the blood stream. The parasites feed upon the contents of the fecal stream and multiply by binary fission of the trophozoite stage. In dysenteric cases, *E. histolytica* characteristically ingests red cells; this has also been reported for *E. coli* when bleeding is present from other causes.

With normal bowel activity, sufficient time elapses for encystment to take place before the parasites are discharged with the feces; thus cysts predominate in formed stools, trophozoites being more often found in the presence of diarrhea. The cysts passed in the feces are the infective agents for new hosts. Those of *Endamoeba histolytica* are susceptible to drying but remain viable in water for several weeks. The trophozoites are fragile animals and quickly die unless kept moist and at the osmotic pressure of tissue fluids. Although experimental infection has resulted from feeding trophozoites to laboratory animals, this stage rarely succeeds in reaching a new host and would normally succumb in the gastric secretions. The method of transmission of *Dientamoeba fragilis,* for which only the trophozoite stage exists, is not known.

Man acquires his infection by ingesting food and drink contaminated with feces containing the mature cystic stage. Since trophozoites predominate in liquid stools the individual with clinical dysentery is a lesser hazard than the infected person passing formed stools which contain cysts. These carriers or "cyst-passers" constitute a large reservoir of infection and are often the direct source of new cases. Serving in various capacities, especially as food handlers, their cysts may reach the food or drink of others. Ivanhoe (1943) has emphasized the importance of direct contact. Transfer may be through uncooked vegetables which have been fertilized with human excreta or washed with polluted water. Epidemics have been traced to contaminated drinking water; such water is rendered potable only by satisfactory filtration or chemical treatment which leaves a residual chlorine of one part per million at the end of 30 minutes. Finally, flies may carry the infective stage from feces to food. Cysts of *E. histolytica* have been recovered from the droppings of flies 48 hours after feeding on infective material.

The ingested cysts pass unharmed through the stomach and some distance down



Life cycle of *Endamoeba histolytica.*

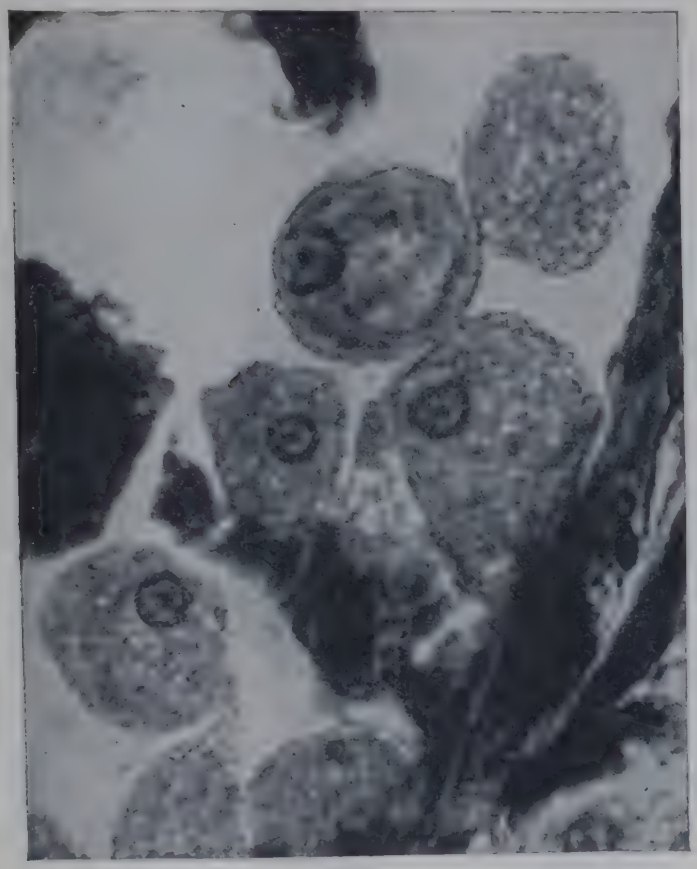
the intestinal tract, probably to the lower ileum, where excystation takes place. The ameba escapes through a pore in the cyst wall and grows and divides to form a colony of trophozoites.

Clinical Illness. Infection with *Endamoeba histolytica* may result in clinical disease or in an asymptomatic carrier state. Only a minority of those infected develop definite pathologic states and but a small percentage of these show serious illness. However, clinical symptoms may later develop in persons who are now

carriers and the asymptomatic infection may be transmitted to others and produce illness. For this reason, all persons with demonstrable infection should receive specific treatment.

Amebiasis must be differentiated from other types of dysentery and should be suspected in any chronic disturbance of bowel function. It has been mistaken for malignancy of the colon; surgical interference due to an error in diagnosis leads to a poor prognosis.

Dientamoeba fragilis has been incriminated as a cause of intestinal symptoms in rare cases, always in the presence of unusually heavy infections (Knoll and Howell,



Nest of trophozoites of *Endamoeba histolytica* in intestinal wall. (Photomicrograph × 1200.)

1945; Perry, 1947). As this organism does not invade tissues, its pathogenicity is debatable. *I. bütschlii* may invade tissues in rare instances (Derrick, 1945).

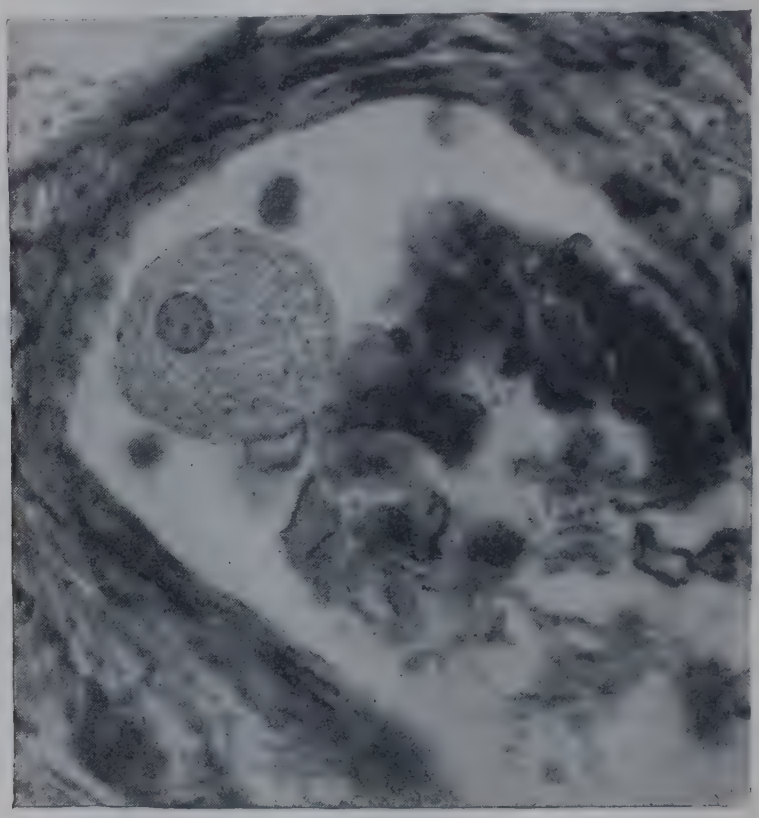
Amebic Dysentery. Named from the predominant clinical feature, amebic dysentery may assume an acute or chronic course. The incubation period may vary from one to several weeks, followed by the development of diarrhea of increasing severity. The stools are copious and may be numerous, with increasing amounts of blood and mucus; abdominal tenderness may be localized, tenesmus is usually absent, and fever and toxemia are mild or absent. The disease tends to develop a chronic course accompanied by weight loss—the walking dysentery. Attacks of constipation may alternate with diarrhea. Characteristic ulcerations are produced in the lower ileum and the colon, the sigmoid and the cecum being favorite sites.

Amebic Colitis (without Dysentery). Symptoms are variable in character and

severity in this type of infection. The usual clinical picture is that of an "irritable colon" but may simulate that of appendicitis or chronic surgical conditions.

Carriers. A majority of those harboring *Endamoeba histolytica* have no symptoms referable to their infection. The carrier state may also develop following recovery from symptomatic infection.

Amebic Abscess. Abscess of the liver is a frequent complication of amebic dysentery; it is occasionally seen without preceding evidence of infection. Involvement of the lung usually develops as an extension from the liver. Abscesses of the brain and other organs and invasion of the skin are less common. *Amebic hepatitis* may be present without abscess formation.



Trophozoite of *Endamoeba histolytica* within an intestinal venule. (Photomicrograph × 1200.)

Other Amebae. A third member of the genus *Endamoeba* lives as a commensal organism in the human mouth: *Endamoeba gingivalis* (Gros, 1849) Smith and Barrett, 1915. The nucleus is characteristic of the genus. Only the trophozoite stage is found, and transfer is probably by direct passage of this stage from host to host. While it appears to be more prevalent in the presence of gingival disease, it bears no causal relation to oral infection.

The Flagellates

Flagellates are less frequently encountered than the amebae in the intestinal tract of man. One species has been generally accepted as being pathogenic; as it does not attack the tissues of the host, however, the pathogenicity of this organism has been questioned by some authors.

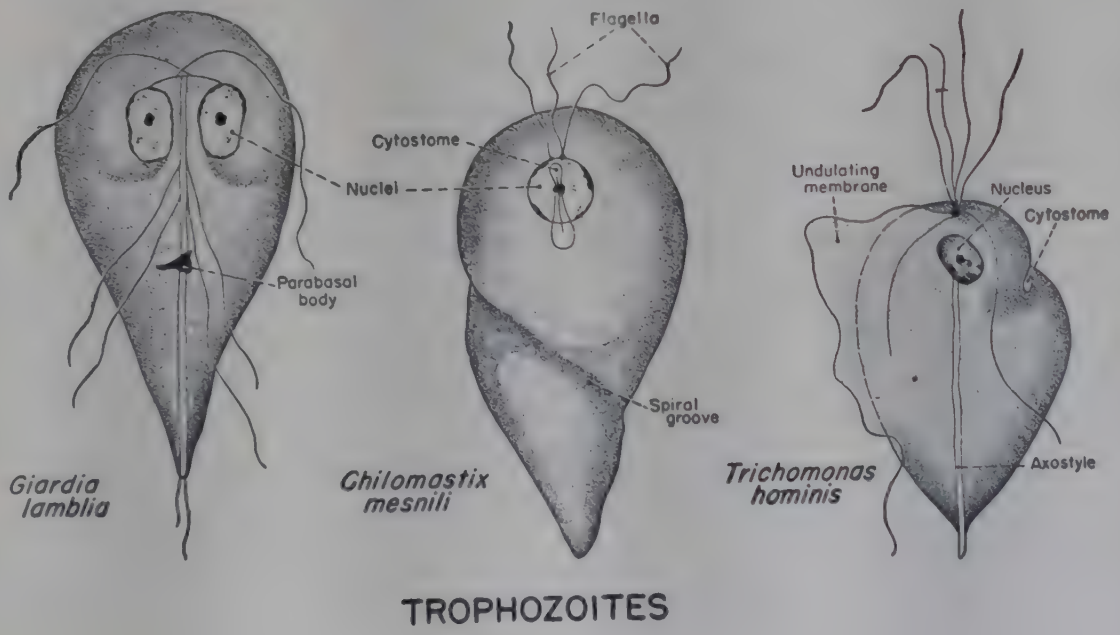
Classification. There are five species of protozoa of the class Mastigophora

that may inhabit the intestinal tract of man. In comparison with the parasitic flagellates previously discussed, these organisms have multiple flagella and may or may not have an undulating membrane. Each of the five species belongs to a separate genus and family.

Giardia lamblia Stiles, 1915.
Chilomastix mesnili (Wenyon, 1910) Alexeieff, 1912.
Trichomonas hominis (Davaine, 1860) Leuckart, 1879.
Enteromonas hominis da Fonseca, 1915.
Retortamonas intestinalis (Wenyon and O'Connor, 1917).

Enteromonas hominis and *Retortamonas intestinalis* are only rarely encountered; the other three species occur with sufficient frequency to require their routine identification. Kirby (1945) has shown the common intestinal trichomonad to have five flagella and has suggested that it belongs to the genus *Pentatrichomonas.*

Morphology. With the exception of *Trichomonas hominis,* these flagellates exist as both trophozoites and cysts; trichomonads are found only as trophozoites.



Trophozoites and cysts of the common intestinal flagellates.

The characteristic motility produced by the lashing flagella facilitates the recognition of the living trophozoites as mastigophorans; it is often diagnostic for the species. The shape and certain other morphologic features are visible in the stained specimens, although *Trichomonas* stains poorly. The cysts also present features which differentiate the species. Nuclei as well as fibrils are visible within them. Mature cysts of *Giardia lamblia* have four nuclei, those of *Chilomastix mesnili* only one.

The trophozoite of *Enteromonas hominis* is rounded or pear-shaped and has four flagella, three anterior and one posterior; the mature cyst is barrel-shaped with nuclei paired at opposite ends. The trophozoite of *Retortamonas intestinalis* is smaller than that of *E. hominis;* it has two flagella anteriorly, and the cysts are pear-shaped with a single nucleus. The small size of the cysts of these two species prevents confusion with pathogenic organisms.

Life Cycles and Transmission. The intestinal flagellates prefer a liquid medium and normally have their habitat high in the intestinal tract; *Giardia lamblia* may be present in the duodenum in large numbers. As with the amebae, the mature cysts discharged in the feces are the infective stages; when ingested by the new host, excystation occurs and the liberated trophozoites establish a colony by longitudinal fission. The method of transfer of *Trichomonas hominis* is not known; however, the trophozoites are unusually resistant and may be capable of serving as the infective stage.

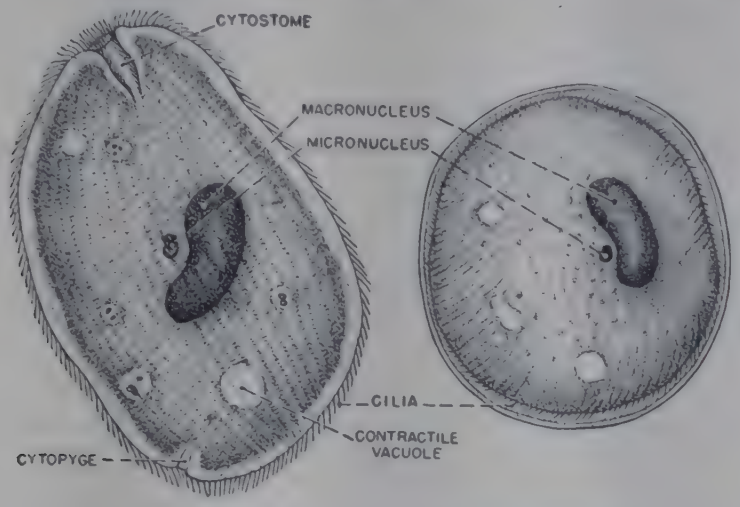
Clinical Illness. Giardiasis is of much less significance than amebiasis. Large numbers of *Giardia lamblia* may be found in certain cases of diarrhea, for which the organism has been considered the etiologic agent. On the other hand, some authorities believe that the incidence of such diarrhea is no higher in those who harbor *G. lamblia* than in those who do not (Monat and McKinney, 1946). Other symptoms of infection include varying degrees of functional disturbance. From its habitat in the duodenum, the parasite may reach the gall-bladder (Lang, 1945). Since the discovery of a specific therapeutic agent (atabrine), there appears to be no question that symptoms are due to the presence of this flagellate; in most instances the infection is entirely asymptomatic. The other intestinal flagellates are nonpathogenic.

Other Flagellates. Two other species of the genus *Trichomonas* are parasites of man: *T. vaginalis* Donné, 1837, usually considered pathogenic, and *T. tenax* (O. F. Müller, 1773) Dobell, 1939, a commensal organism of the human mouth. Only the vegetative stage is recognized and transmission from host to host requires contact sufficiently direct to transfer the viable trophozoite. These trichomonads show only slight morphologic differences when compared with *T. hominis: T. vaginalis* is larger and has four anterior flagella and a short undulating membrane; it has no free posterior flagellum.

Trichomonas vaginalis is associated with a specific vaginitis accompanied by a profuse and irritating discharge; the vaginal mucosa becomes inflamed and congested. The flagellate may also be found in a chronic urethritis in the male. Bacterial infection is frequently present and may initiate or aggravate the symptoms.

The Ciliate

Only one ciliate is parasitic in man. This organism causes a specific disease, *balantidiasis,* infrequently seen but producing ulcerative lesions of the colon and lower ileum and having a relatively high fatality rate. The disease has recently



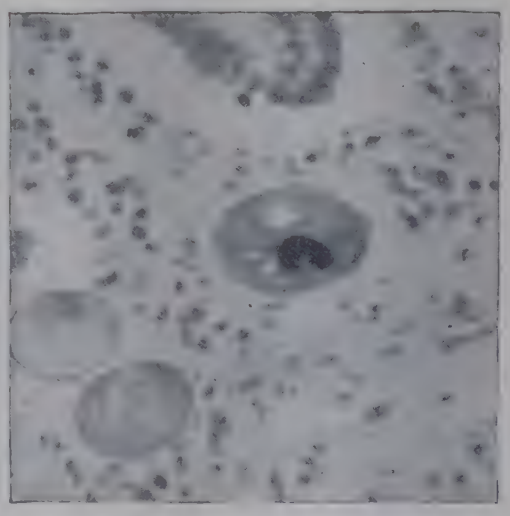
Trophozoite and cyst of *Balantidium coli.*

been successfully treated with stovarsol (Dias Atiles, 1943) and carbarsone (Tsuchiya and Kenamore, 1945). The infection may be encountered in any part of the world.

Classification. Most of the protozoa of the class Ciliata (Infusoria) are free-living aquatic forms. The single species found in man belongs to the order *Heterotrichida,* differentiated from others by the arrangement of the cilia around the cytostome. Related species are found in other animals.

Balantidium coli (Malmsten, 1857) Stein, 1862.

Morphology. *Balantidium coli* is a large protozoan, 50 by 70μ, barely visible with the naked eye. The trophozoite is oval in shape and covered with cilia which are in constant motion when the animal is alive. Cysts are less commonly found; they are smaller than the trophozoites and spherical in shape.



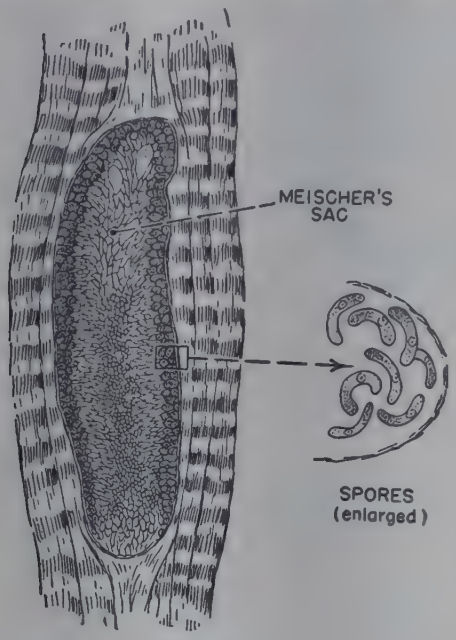
Trophozoite of *Balantidium coli* in intestinal wall. (Photomicrograph × 300.)

Life Cycle and Transmission. Ciliates identical with those infecting man are common parasites of hogs and may be found in monkeys. Hogs and man, therefore, serve as a reservoir of infection, passing the infective cysts in the feces. The ingested cysts release active trophozoites which select a habitat in the bowel where the environment is fluid or semifluid. Reproduction is by binary fission of the trophozoites. Occasionally this follows conjugation, two animals temporarily fusing and exchanging nuclear material, then again becoming separate individuals.

Clinical Illness. The trophozoites of *Balantidium coli* may penetrate the intestinal mucosa with resulting necrosis and ulceration. This is accompanied by secondary bacterial infection, the ulcers extending laterally and penetrating the deeper layers of the intestinal wall. Hemorrhage and inflammatory exudate result.

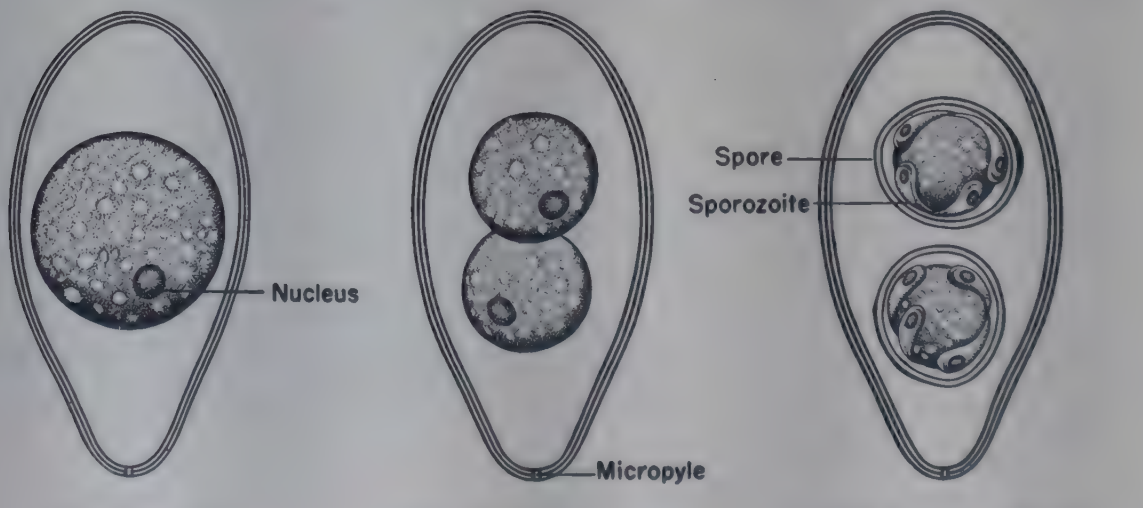
The Sporozoans

In addition to the malaria parasites, other sporozoans may infect man. Only one is an intestinal parasite, and this organism, *Isospora hominis* (Rivolta, 1878) Dobell, 1919, is rarely or questionably pathogenic for man. However both Liebermann (1945) and Humphrey (1946) report cases in which they believe this organism caused definite pathologic lesions. Closely related organisms are common in dogs and cats. A second sporozoan, *Eimeria stiedae,* is a parasite of the rabbit, and it, or a closely related species, has been reported as infecting man in a few cases.



Sarcocystis in muscle tissue.

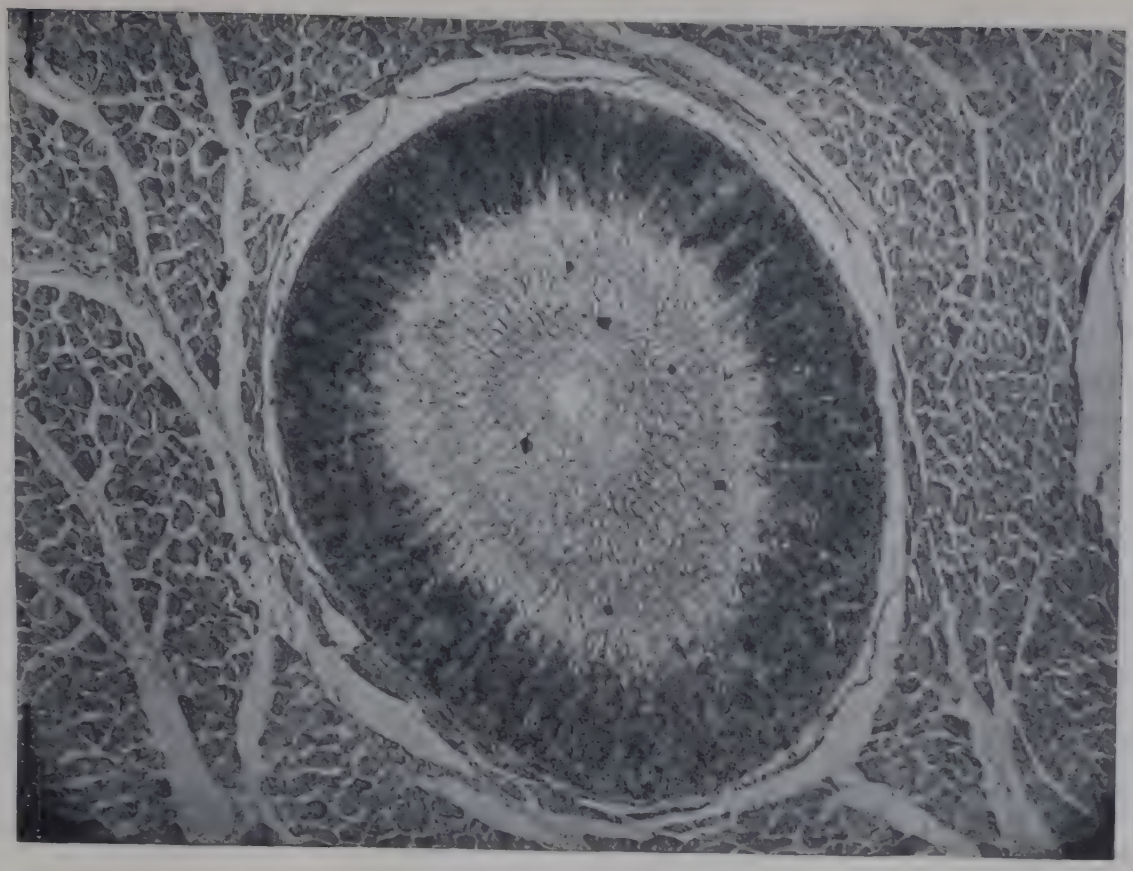
These two parasites belong to the order *Coccidia.* The protozoans of this group have life cycles similar to that of malaria but with the alternation of generations within the same host. Both sexual and asexual cycles develop in epithelial cells. Sporogony continues outside the body of the host when the oöcyst is passed in the feces; ingested spores rupture and liberate sporozoites which invade the cells of the new host.



Oöcysts of *Isospora hominis.* (Redrawn from Dobell and O'Connor, 1921.)

Parasites of Uncertain Relationships

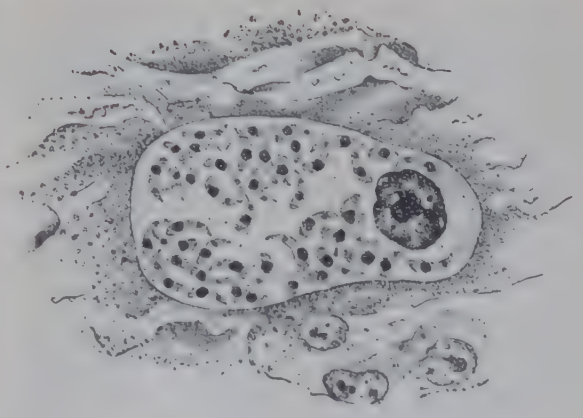
Sarcocystis. This organism in man may be identical with one or more species of this genus found in other animals. Infection in man is asymptomatic, being discovered at autopsy. The parasites encyst within striated muscle as sarcocysts (Miescher's tubes)—



Sarcocystis. (Photomicrograph × 150.)

elongated tubular bodies containing spores. The life history is unknown, but mice may be infected by feeding them spores. The work of Spindler (1945) indicates that this organism is a fungus and not a protozoan.

Toxoplasma. This is a protozoan not readily classified. Infection in man is uncommon, usually seen in young infants in whom it produces an encephalitis. The parasites are found at autopsy in the reticulo-endothelial cells where they superficially resemble *Leishmania* or *Sarcocystis*. The organisms may be demonstrated in impression smears stained as for malaria. They may be isolated by injecting suspected material into albino mice, guinea pigs, or rabbits. Antibodies may be detected by neutralization tests or complement fixation. Toxoplasmosis has recently assumed greater importance owing to the discovery of a number of cases in the United States (Weinmann, 1944; Heath and Zuelzer, 1945; Callahan, 1945; Miller, 1947).



Toxoplasma.

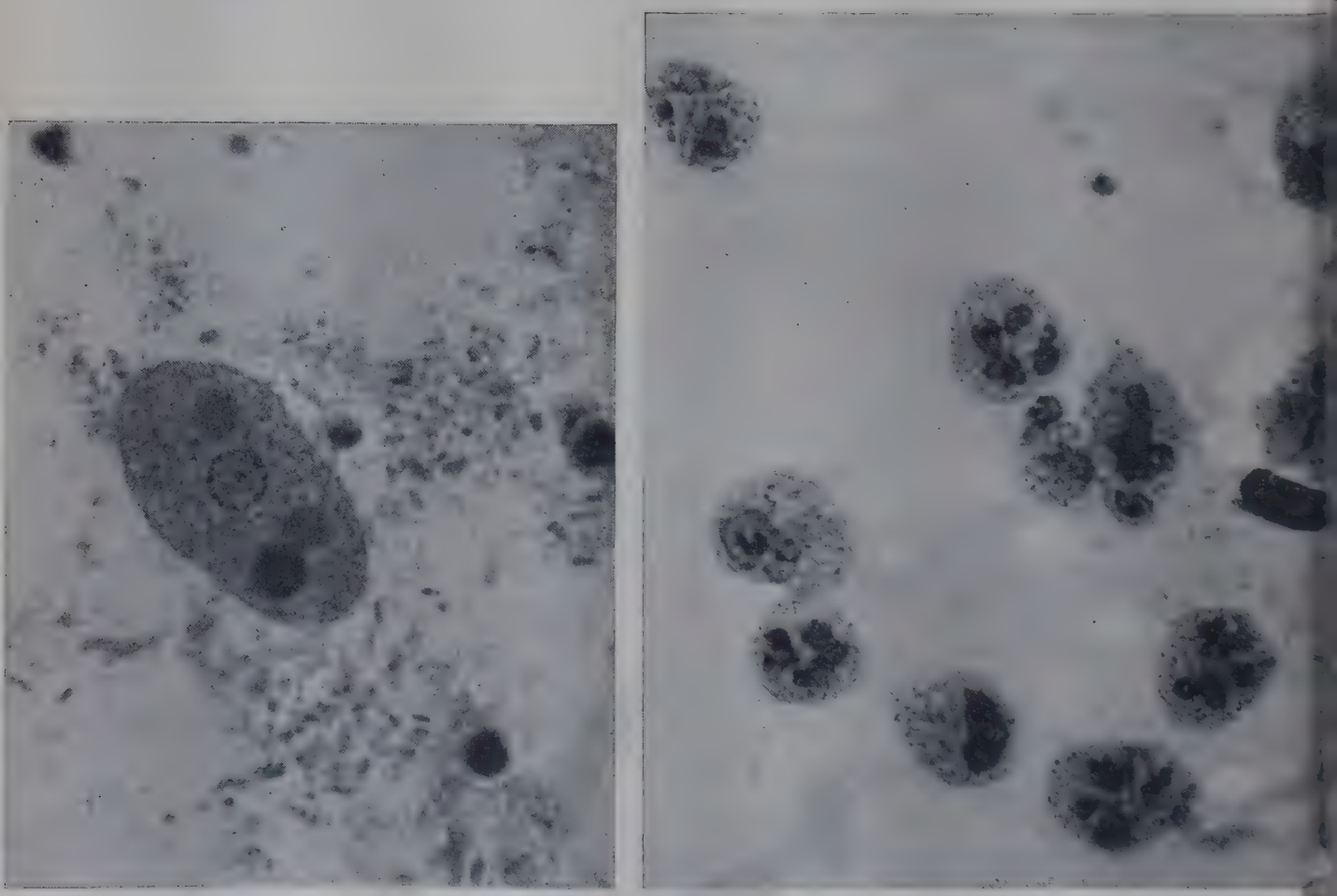
Diagnosis of Intestinal Protozoa

The gross and microscopic appearance of the stools in acute dysentery is of value in differentiating amebic from bacillary infections. In amebic dysentery the stools are usually fluid, relatively copious, and contain fecal material, much blood-streaked or brownish mucus, and varying amounts of fresh or altered blood which gives them a dark-brown or reddish color. They are often fetid. This contrasts with the scanty, watery, nonfecal bowel movements containing masses of white mucus flecked with bright-red blood in the bacillary type. Microscopically, the

amebic stools show mucus and numerous red cells, often clumped and degenerated, but very few pus cells or phagocytic cells which are numerous in bacillary dysentery. The white cells which are present mostly show cytolysis and consist of scanty, ragged cytoplasm surrounding pyknotic nuclei. Charcot-Leyden crystals are highly suggestive but not pathognomonic of amebic dysentery, being found in other conditions which produce bowel hemorrhage.

A diagnosis of infection with intestinal protozoa is confirmed by demonstrating the specific organisms in the feces, in tissues, or in material from other sources. Primary interest centers around the identification of the pathogenic species which, however, must be differentiated from the nonpathogenic forms which they resemble. Trophozoites will be found in liquid or mushy stools, being generally absent in the formed feces. Those of *Endamoeba histolytica* may be recovered from the flecks of mucus adherring to the formed stools, in material aspirated or draining from an amebic abscess, from the base of specific ulcers, or in sputum when there is pulmonary involvement; cysts are not present in such material. Sigmoidoscopy and proctoscopy in the hands of trained investigators often yield valuable results (Cropper, 1945; Rail, 1946). Because of their normal habitat in the duodenum, trophozoites of *Giardia lamblia* are usually seen only in the presence of diarrhea.

The cysts are often of more aid to the diagnosis than the trophozoites; in the



(*Left*) Characteristic fecal smear from case of amebic dysentery, showing trophozoite of *Endamoeba histolytica* with ingested red blood cells. (*Right*) Characteristic fecal smear from case of bacillary dysentery, showing numerous white blood cells. (Photomicrographs × 1200.)

presence of diarrhea it may be necessary to reduce bowel activity in order to obtain cysts from which to make a diagnosis. On the other hand, catharsis may be necessary in order to recover *Dientamoeba fragilis* and *Trichomonas hominis* which exist in only the trophozoite stage.

In searching for the protozoa it is usual to start with a fresh smear of the feces or other material which may contain them. This is examined as a wet preparation made in isotonic solution to detect the characters of living trophozoites and cysts. Having demonstrated the presence of protozoans and some of their features, additional information may be obtained by using an aqueous smear and an iodine stain. Final confirmation and details are developed by a fixed smear stained with iron-hematoxylin. Laboratory procedures which depend upon concentration technics or stained smears exclusively lose the benefit of some of the characters seen in the living forms. Culture methods are advocated by some workers (Lucas, 1945). Roentgenograms may show early evidence of amebiasis (Hodes and Mammoser, 1947).

Trophozoites and Cysts in Isotonic Solutions (The Normal Saline Smear). Under low magnification (16-mm. objective) and in isotonic solution the living trophozoites of the amebae appear as highly refractile, shining bodies varying from the size of the smallest pinhead to that of a buckshot. The 4-mm. objective discloses the clear, homogeneous ectoplasm and pseudopodia; the granular endoplasm and sometimes a nucleus can be seen.

The cytoplasm of the cysts is less granular than that of the trophozoites; the cyst wall often has the appearance of a steel wire in optical section. Cysts are more refractile than trophozoites. Their smooth outline and colorless appearance, at the most tinted faintly blue or green, under low power marks them as protozoans.



Cysts of *Endamoeba coli* in iron hematoxylin-normal saline smears. (*Left*) Note high refractility and faultlessly smooth cyst wall. (*Right*) Note nuclei. (Photomicrographs × 150.)

Trophozoites of the flagellates and the ciliate are recognized by their motility. Their cysts resemble those of the amebae in their smooth outline but are somewhat less refractile. Additional specific features which may be visible have been described under morphology. The oöcyst of the sporozoan is highly refractile with characteristic shape and double wall.

Having identified the presence of trophozoites or cysts, these may be examined further for specific diagnostic features.

Size. Cysts of each species are more uniform in size than the trophozoites. Either may present a rather wide range, but the majority of any species varies only 2 or 3μ. The exception is *Endamoeba histolytica,* trophozoites ranging from 8μ, small race, to an average of 24μ in dysenteric cases. *Balantidium coli* is above the range of the other intestinal protozoa. (See Tables 55 and 56.)

Shape. The ameba trophozoites present various contours, depending upon their pseudopodial activity; the shapes of the flagellates and the ciliate are often diag-

Table 55

SIZE OF TROPHOZOITES

Small (under 10μ)	*Medium to Small (10–12μ)*	*Medium (12–14μ)*	*Large (18 or more μ)*
E. histolytica (small race)	*I. bütschlii*	*E. histolytica* (nondysenteric)	*E. histolytica* (dysenteric)
E. nana	*D. fragilis*	*C. mesnili*	*E. coli*
		G. lamblia	

Table 56

SIZE OF CYSTS

Small (under 10μ)	*Medium (about 12μ)*	*Large (about 18μ)*	*Variable (6–16μ)*
E. histolytica (small race)	*E. histolytica* (large race)	*E. coli*	*I. bütschlii*
E. nana	*E. coli* (small)		
C. mesnili	*G. lamblia*		

nostic. Many of the cysts have a characteristic shape which aids in their identification, especially when considered with size (see Table 58, p. 541).

MOTILITY. The motility most commonly seen in the amebae consists of apparently purposeless extensions and retractions of ectoplasm in various directions. These pseudopodia may be tonguelike, crescent-shaped, budding, or knoblike. In two instances the pseudopodial activity is distinctive and of diagnostic value: (1) the tonguelike, explosive pseudopod giving a progressive directional crawl to *Endamoeba histolytica;* (2) the thin, fanlike pseudopod with sharp corners and points of *Dientamoeba fragilis.* In the type of motility described as "progressive directional crawl" there is a rapid succession of ectoplasmic projections mainly in one direction. The animal becomes elongated and ribbon-like with an anterior advancing and a posterior dragging end to which a brush of debris is frequently attached. As the ectoplasm advances the endoplasm flows forward. Sometimes these motions become so lively that the action appears continuous. When this type of motility is seen it identifies the trophozoite as *E. histolytica,* with very rare exceptions; *E. coli* may imitate this movement, but is normally sluggish and rarely directional. *Endolimax nana, Iodamoeba bütschlii,* and *Dientamoeba fragilis* show little or no progression or are never directional.

The motility of the common flagellates and the ciliate is distinctive for each genus. *Giardia* has a slow, tumbling, or falling-leaf movement. *Chilomastix* moves by boring ahead with slow rotation of the body. *Trichomonas* moves by means of quick, nervous, repeated jerks, apparently making little progress. *Balantidium coli* moves steadily across the field with visibly beating cilia and a slow rotary movement of the body.

Visible Structures and Inclusions. Internal structures may be visible in both trophozoites and cysts, and from them a diagnosis may be made. The nucleus of the *Endamoeba coli* trophozoite is visible; it is rarely so in *E. histolytica.* Trophozoites of *Giardia,* when immobile, will show nuclear rings, the axostyle, and the tail; those of *Chilomastix* show the spiral groove encircling the body; *Trichomonas* may be identified by the lashing flagella and the undulating membrane. The cilia, contractile vacuole, and kidney-shaped macronucleus of *Balantidium coli* are diagnostic. Ingested erythrocytes may be considered diagnostic for *E. histolytica;* to avoid confusion with the rare ingestion of red cells by *E. coli* the diagnosis may be confirmed by the characteristic motility or by the identification of cysts. The endoplasm of *E. histolytica* does not contain ingested bacteria and food particles which are usually seen in other ameba trophozoites. There are also minor differences such as the uniformity of the endoplasm of *E. histolytica* and its thinness in *Dientamoeba fragilis,* but there are many exceptions to these.

The cysts also present structural features of diagnostic value. When nuclei can be seen their number may aid in species diagnosis; only *Endamoeba coli* has more than four. Most important are the chromatoid bodies found in about one-half of the cysts of *E. histolytica.* These appear as highly refractile rods or bars with smooth or rounded ends. In contrast, chromatoid bodies are much more rarely found in *E. coli* and appear as strands or bars with splintered ends. In the absence of chromatoid material, living cysts of small race *E. histolytica* and of *Endolimax nana* are indistinguishable. Cysts of *Iodamoeba bütschlii* show a clearly defined glycogen mass and clusters of volutin granules. Those of *Giardia* have a narrow, clear space between the cyst wall and the encysted animal; in addition, nuclei, fibrils, and rods are visible within the cytoplasm.

While it is always well to confirm by staining, a positive identification of species can be made from living trophozoites and cysts by an experienced technician as follows:

Endamoeba histolytica:
Trophozoites with ingested red blood cells.
Cysts containing rod-shaped chromatoid bars.
Endamoeba coli:
Cysts containing more than four visible nuclei.
Iodamoeba bütschlii:
Cysts containing a visible glycogen "ball."
Dientamoeba fragilis:
Trophozoites when motile.
The flagellates and ciliate:
Trophozoites showing typical shape and motility.
Cysts showing typical shape, size, and visible structures.

Trophozoites and Cysts in Hypotonic Solutions (The Aqueous Smear). Smears made with hypotonic solutions may be used to distinguish rounded-up

Table 57

CHARACTERS OF LIVING TROPHOZOITES

Species	*Size (in microns)*	*Shape (at rest)*	*Other Diagnostic Features*
Endamoeba histolytica	Range 6–40 (see text)	Rounded	Progressive directional crawl. Ingested red blood cells; "clean" endoplasm
Endamoeba coli	Range 12–30 (average 20)	Rounded	Sluggish; broad pseudopodia. Visible nucleus; "dirty" endoplasm
Endolimax nana	Range 6–12 (average 8)	Rounded	Sluglike; not progressive. Nucleus rarely visible
Iodamoeba bütschlii	Range 6–20 (average 10)	Rounded	Not progressive; nucleus rarely visible
Dientamoeba fragilis	Range 6–18 (average 12)	Faultlessly spherical	Thin cytoplasm, visible inclusions, invisible nuclei
Trichomonas hominis	Usually less than 10	Ovoid	Nervous, jerking movements. Undulating membrane, flagella
Giardia lamblia	Range 12–15	Pear-shaped	Tumbling, falling-leaf motion. Visible nuclei, axostyles
Chilomastix mesnili	Range 12–15	Cone-shaped	Visible spiral groove. Boring, progressive movement
Balantidium coli	Average 50 × 70	Oval	Steady progress, slowly rotary. Visible: waving cilia, cytostome, nucleus, vacuole

trophozoites from cysts, the former being destroyed. The characteristic disintegration of *Dientamoeba fragilis* when exposed to reduced osmotic pressure provides a useful method of identification. By the time the smear made with tap water or distilled water is placed under the microscope the trophozoites have begun to distend. The endoplasm becomes thin, lusterless, and transparent. Bacteria and other inclusions become clearly visible; the nucleus or nuclei can be seen with good illumination as dully refractile discs. The distention terminates in 1 to 10 minutes with an explosive rupture of the ectoplasm and expulsion of its contents. The ectoplasm then, like a rubber ball, quickly regains its spherical form, remaining intact for several minutes. Some particles, and occasionally a nucleus, may remain within the shell (Hakansson, 1937).

The aqueous smear is routinely used to eliminate blastocysts. Many fecal specimens contain this vegetable organism, and it is the source of much annoyance and many mistakes. The blastocysts distend and disintegrate in the hypotonic solution, leaving a clear field for protozoan cysts that may be present; some do not rupture immediately, but all quickly lose their luster and can be readily differentiated from the refractile cysts.

Trophozoites and Cysts in Stains (The Iodine Smear). Iodine is used to stain the chromatin material of the nuclei and the glycogen; it kills both tropho-

Table 58

CHARACTERS OF LIVING CYSTS

Species	*Size (in microns)*	*Shape*	*Other Diagnostic Features*
Endamoeba histolytica	Range 5–16 (average 8 or 12)	Round	Chromatoid bars, rounded ends. Nuclei rarely visible
Endamoeba coli	Range 10–30 (average 17)	Round	Chromatoid material rarely seen. More than 4 visible nuclei
Endolimax nana	Range 6–10 (average 8)	Oval and round	None
Iodamoeba bütschlii	Range 6–16 (average 10)	Odd shapes or rounded	Definite glycogen "ball." Clusters of volutin granules
Dientamoeba fragilis	No cysts		
Trichomonas hominis	No cysts		
Giardia lamblia	Average (8 by 12)	"Football" (round, on end)	Clear space inside cyst wall. Fibrils, rods, rings
Chilomastix mesnili	Average 8	Lemon-shaped	Nipple-like thickening. Nucleus and fibrils not visible
Balantidium coli	Range 50–60	Round	Thick double wall. Kidney-shaped macronucleus visible

zoites and cysts. Such staining frequently fails to bring out the finer details on which the species of amebae are differentiated, merely showing the number and arrangement of the nuclei. The nuclei of young cysts of *Endamoeba histolytica* and *E. coli* may be obscured by the more densely stained glycogen. Iodine will arrest the activity of the flagellates and the ciliate, aiding in the visualization of these trophozoites; otherwise, it is of aid in identifying only:

Endamoeba histolytica, large race—Cysts (nuclei).
Endamoeba coli—Cysts (nuclei).
Iodamoeba bütschlii—Cysts (glycogen ball).

Trophozoites and Cysts Fixed and Stained (The Iron-Hematoxylin Smear). While the oil-immersion lens is of little value in wet preparations, it is essential in the examination of stained smears. As stained with iron-hematoxylin the details of the nuclei and other structures may be observed. The glycogen is lost in the staining procedure but the vacuole remains. This is a permanent stain and produces the best differential characters, summarized in Table 59 and discussed in detail in the following pages. The organisms retain the shape described for the living forms; size is reduced slightly owing to shrinkage in fixation.

ENDAMOEBA HISTOLYTICA. Two races or strains of this ameba are recognized: a large race, cysts averaging 12μ, and a small race, cysts averaging 8μ. Trophozoites may be found in liquid stools or in mucus. Immature cysts are almost exclusively uninucleate.

1. A trophozoite from a nondysenteric case ("carrier"). The chromatin granules on the nuclear membrane are uniform.

2. A trophozoite in amebic dysentery, although there are no ingested red cells. The parasite is over 16μ in length. The small karyosome is centrally placed.

3. A giant trophozoite containing ingested red cells in various stages of absorption. The nucleus is characteristic.

4. An immature, uninucleate cyst (a very common type). The chromatoid matter is scattered and the outline of the glycogen vacuole is visible.

5. An immature, uninucleate cyst with a large nucleus and a definite glycogen vacuole (a very common type). Note that the nucleus is not crowded by the vacuole. Chromatoid grains are scattered around the periphery of the cyst.

6. An immature cyst in which the nucleus is dividing (rare).

7. An immature cyst with two nuclei (rare). The glycogen is less definite but the chromatoid matter is forming into bars and chunks.

8. A trinucleate cyst, one nucleus being larger than the other two (rare). A single chromatoid bar with rounded ends is present.

9. A mature, quadrinucleate cyst (a very common type). The glycogen has been absorbed; the nuclei are characteristically placed. The chromatoid bar is diagnostic.

10. A mature cyst with characteristic nuclei and chromatoid bars (a very common type). Note the vacuolization.

11. A mature cyst, larger than the average for this species. The chromatoid matter has been absorbed.

12. A trophozoite, small race. The granules on the nuclear membrane are uniform and the small karyosome centrally placed.

13. An immature, uninucleate cyst, small race. There is a large amount of chromatoid matter, the best diagnostic aid in the small-race cysts.

14. An immature, binucleate cyst with chromatoid bars. The glycogen is not so definite as seen in the large-race cysts.

15. A mature, quadrinucleate cyst with two chromatoid masses, one seen on end.

16. A mature, quadrinucleate cyst in which the chromatoid matter has been absorbed.



Endamoeba histolytica. Drawings made from actual specimens observed in iron-hematoxylin stained, Schaudinn fixed, fecal smears.

ENDAMOEBA COLI. Small races of *Endamoeba coli* are uncommon; it is usual to find these forms significantly larger than those of the other amebae. Only the giant *E. histolytica* trophozoites in amebic dysentery attain the size or surpass that of *E. coli*. In this species the characteristic immature cyst is binucleate while that of *E. histolytica* is uninucleate. Ninety-five per cent of the cysts of *E. coli* are either two-nucleate or eight-nucleate, the predominating number depending upon the consistency of the feces.



Endamoeba coli. Drawings made from actual specimens observed in iron-hematoxylin stained, Schaudinn fixed, fecal smears.

1, 2. Two trophozoites, about 25 or 30μ long respectively. The karyosome is large and eccentrically placed; the chromatin granules on the nuclear membrane are deposited irregularly. The endoplasm is profusely vacuolated and dirty with visible food inclusions.

3. An abnormally large, uninucleate, immature cyst (rare). The glycogen vacuole is large and conspicuous. Many authorities prefer to call this form a precystic stage.

4. A young binucleate, immature cyst, about 17μ in diameter (rare).

5. The characteristic immature, binucleate cyst (a very common type). The nuclei assume diametric positions, crowded against the cyst wall by the glycogen vacuole. Scattered chromatoid granules surround the vacuole.

6. An immature, quadrinucleate cyst (rare). The nuclei, each of which must undergo another division, are much larger and more centrally placed than in the quadrinucleate cyst of *E. histolytica*.

7. An immature five-nucleate cyst (rare). The three larger nuclei must undergo another division.

8. An immature, six-nucleate cyst (rare). The two larger nuclei have not undergone final division. The majority of the chromatoidal material has been absorbed, not unusual in immature cysts of *E. coli*.

9. A mature, eight-nucleate cyst (a common type). The spindle-shaped, sharply pointed chromatoid bodies are distinctive for *E. coli*.

10. A mature cyst with filamentous chromatoid bodies, commonly seen in this species.

11. A mature cyst with only a small speck of chromatoid matter remaining (a common type).

12. A mature cyst without chromatoid matter (a very common type).

Table 59

IRON-HEMATOXYLIN STAINED PROTOZOA

Species	*Diagnostic Features*
Endamoeba histolytica	The *histolytica* nucleus. Trophozoites with ingested red cells, "clean" cytoplasm. Cysts with 1 to 4 nuclei, 1 and 4 predominant; chromatoid bars with rounded ends
Endamoeba coli	The *coli* nucleus. Trophozoites with "dirty" vacuolated cytoplasm. Cysts with 1 to 8 nuclei, 2 and 8 most common; chromatoid splinters with sharp ends occasionally seen
Endolimax nana	The *Endolimax* nucleus. Trophozoites uninucleate, may resemble *Iodamoeba bütschlii* except for size. Cysts with 2 or 4 dotlike nuclei
Iodamoeba bütschlii	The *Iodamoeba* nucleus. Trophozoites may resemble *E. nana* except for size. Cysts with the vacuole of the glycogen ball
Dientamoeba fragilis	The *Dientamoeba* nucleus. Only trophozoites present. 1 or 2 nuclei
Giardia lamblia	Trophozoites binucleate, cysts quadrinucleate. Visible flagella, axostyle, blepharoplast, and parabasal body
Chilomastix mesnili	Trophozoite with visible flagella and spiral groove. Cysts uninucleate with visible fibrils in shape of safety pin
Trichomonas hominis	Only trophozoites. Visible undulating membrane, flagella, and axostyle. Often stains poorly
Balantidium coli	The *Balantidium* nucleus. Trophozoite with visible cilia, cytostome. Cyst with double wall

Endolimax Nana. This is a small ameba, both trophozoites and cysts being within the size range of *Endamoeba histolytica,* small race. The cysts have no chromatoid bodies, and usually more than half of them are oval in shape.

1. A trophozoite with a large, prominent karyosome. The nuclear membrane is not visible. The small, black granules are ingested microörganisms lying in food vacuoles.

2. An immature, uninucleate cyst (rare).

3. An immature, binucleate cyst (rare). The karyosome of one nucleus is split.

4. An immature, trinucleate cyst (rare). Note ovoid shape.

5. A mature, quadrinucleate cyst (a very common type). The nuclear membrane is faintly visible. Oval cysts seen on end will present this rounded shape.

Iodamoeba Bütschlii. This ameba is generally larger than *Endolimax nana,* but smaller than *Endamoeba histolytica,* large race, and *E. coli.* The trophozoite may appear exactly like that of *E. nana,* but this is of little importance as neither is pathogenic. The diagnostic nucleus is brought out by staining. In addition the cysts show a sharply defined glycogen mass and clusters of volutin granules, and are of irregular shape.

6. A trophozoite. The black granules in the endoplasm are food inclusions.

7. A young cyst with a characteristic glycogen vacuole and volutin granules (a very common type).

8, 9. Two cysts of different size and shape (common types). They show the typical basket-like nucleus formed by the eccentric karyosome and the opposing granular layer. Glycogen vacuoles and clusters of volutin granules are present in both.

Dientamoeba Fragilis. This ameba has a wide range in size, only trophozoites being found. From 20 to 80 per cent are binucleate forms.

10, 11. Uninucleate trophozoites. The karyosomes are made up of distinct granules, one in triangular and one in circular pattern.

12, 13. Binucleate trophozoites. The nuclear membrane is visible in one.

Endamoeba Gingivalis. Only trophozoites are found. The nucleus is typical of the genus, more closely resembling that of *Endamoeba coli.* The endoplasm is filled with food vacuoles containing dark-staining bodies from ingested food material.



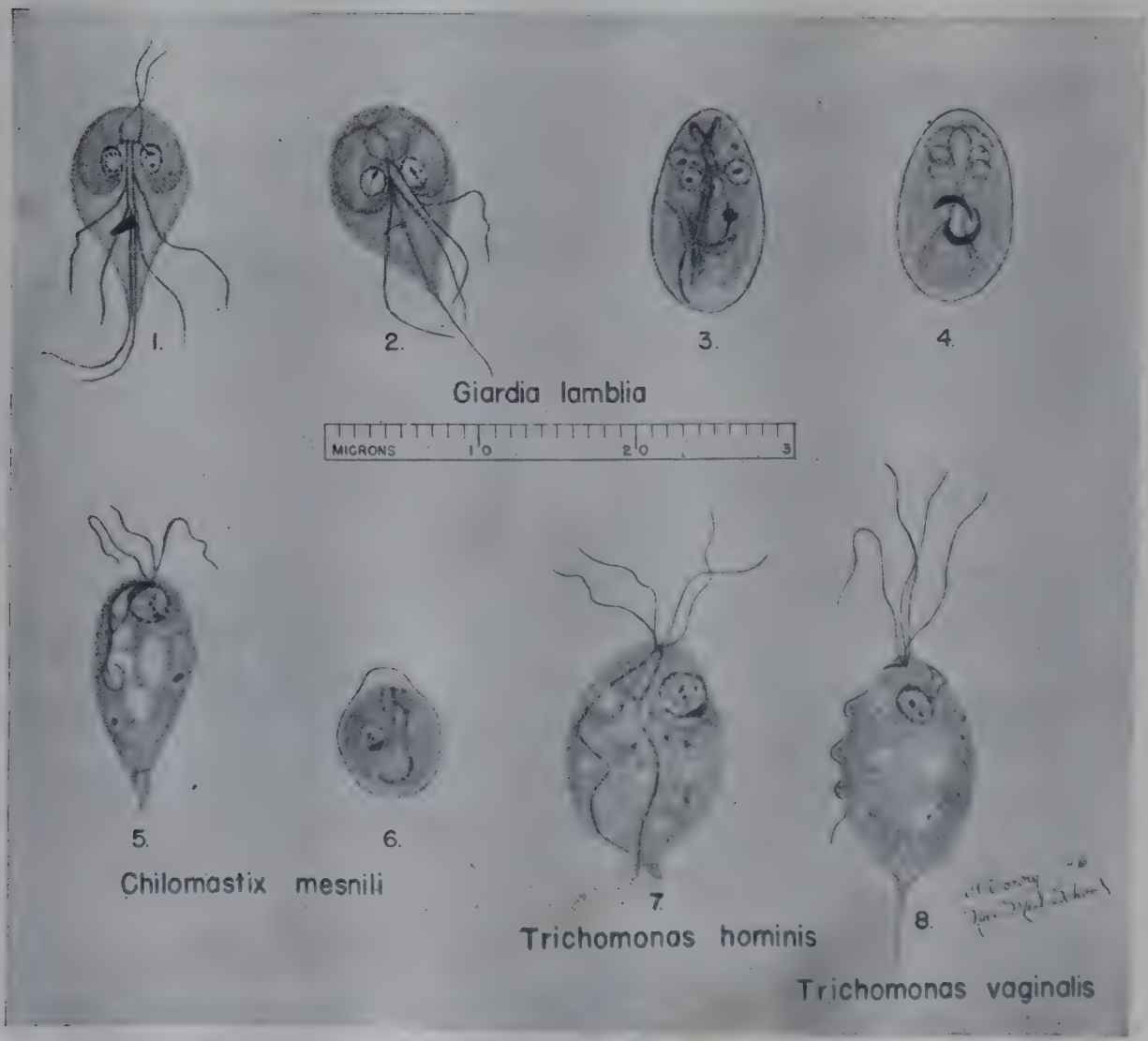
Endolimax nana, Iodamoeba bütschlii, Dientamoeba fragilis, and *Endamoeba gingivalis.* Drawings made from actual specimens observed in iron-hematoxylin stained, Schaudinn fixed smears.

FLAGELLATES: GIARDIA LAMBLIA. This pear-shaped flagellate has a very distinctive appearance. The organelles are in pairs and arranged symmetrically. The trophozoites (1 and 2) are more complete than those usually seen; flagella may or may not be visible. All have two nuclei. Cysts (3 and 4) have four nuclei arranged in pairs at the anterior pole. The fibrils and curving rods, parts of axostyles and flagella, may resemble the lacing of a football.

CHILOMASTIX MESNILI. The body of the trophozoite (5) appears as a long cone; the spiral groove is often visible. The nucleus is vesicular and contains a small karyosome. Flagella are not always seen. The lemon-shaped cyst (6) has the wall at one end thickened into a nipple-like structure. The nucleus resembles that of the trophozoite. Visible fibrils may be described as assuming the shape of a safety pin.

TRICHOMONAS HOMINIS AND T. VAGINALIS. These two trichomonads are very similar, showing only minor differences in shape and morphology. They often stain poorly, making it difficult to recognize the undulating membrane and the number of flagella. Only trophozoites occur.

CILIATES: BALANTIDIUM COLI. The cilia of the trophozoites are usually visible in



Flagellates of the alimentary and genitourinary tracts of man. Drawings made from actual specimens observed in iron-hematoxylin stained, Schaudinn fixed smears.

the stained smears. When properly rotated the cytostome can be seen. The large nucleus with its characteristic shape is always visible; the micronucleus may be obscured. The cyst has a very thick wall; the macronucleus remains visible. The characteristic shape of the nucleus may be lost in tissue sections.

Trophozoites and Cysts Demonstrated by Other Procedures. Only trophozoites are found in tissues; rarely do those of *Endamoeba histolytica* show ingested red blood cells. Again they are identified by the *Endamoeba* nucleus and the granular endoplasm. Cysts obtained by concentration technics may be examined in wet smears and after fixation and staining. Most of the protozoa may be cultivated on suitable media. A complement-fixation test has been developed but has limited diagnostic value.

Related Protozoa. The procedure for the diagnosis of protozoa of the mouth or genitourinary tract is the same as that for the intestinal protozoa. The organisms are found in a fluid medium and it may or may not be necessary to dilute this material. *Endamoeba gingivalis* must be differentiated from *E. histolytica* in the sputum by characteristic motility, appearance of the endoplasm, and nuclear structure. The demonstration of flagellates in vaginal or urethral secretions is usually sufficient for the diagnosis of *Trichomonas vaginalis*.

Objects Resembling Intestinal Protozoa. *Blastocystis hominis* is present in many stools, especially those that are mushy. Under the low magnification used for searching the wet smear, blastocysts are extremely difficult to differentiate from protozoans; they may be confusing under the 4-mm. objective. The majority, however, have a more greenish tint than true cysts. They consist of a central structureless mass surrounded by a clear hyaline layer containing refractile granules; the latter are important identifying features. Even when stained, blastocysts may cause mistakes as the darkly stained granules may appear like nuclei or chromatoid bodies.

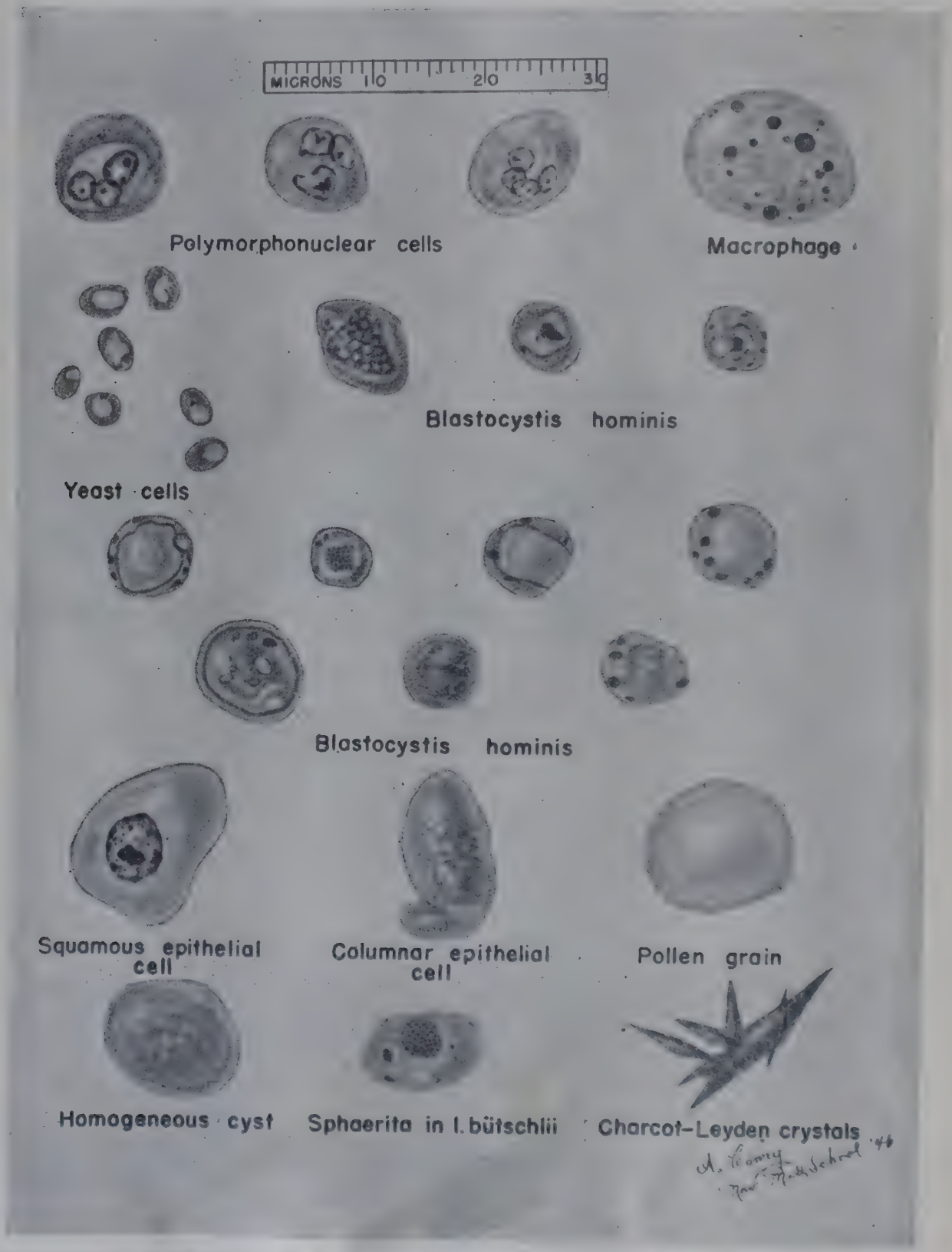
In the presence of pus cells no object should be identified as a trophozoite of *Endamoeba histolytica* unless it is moving. *Macrophages* may be especially confusing since they superficially resemble rounded-up vegetative forms of amebae. They are commonly present in the mucopurulent discharge of bacillary dysentery and may lead to a serious error in diagnosis. *Polymorphonuclear and smaller pus cells* are usually not mistaken for amebae in the wet smears. The *segmented neutrophil* with several nuclear rings may be easily mistaken for a cyst when stained. *Epithelial cells* are less confusing.

Yeasts occur in several forms, the commonest being a small species, 4 to 6μ long and ovoid in shape. They may be mistaken for cysts of *Endolimax nana* in wet smears.

Homogeneous cysts are rounded in shape, varying in size from about 5 to 30μ or more. While some may be colorless, most have a definite yellow tint. This color and the absence of nuclei and other organelles distinguish them from protozoans.

Charcot-Leyden crystals are frequently found in amebic dysentery but also occur in certain other diseases. They are whetstone-shaped, colorless when unstained but black in iron-hematoxylin preparations.

Starch granules that have escaped digestion may be found in the feces. Their size and refractility are the same as those of the protozoan cysts in wet smears. Under higher magnification they may be recognized by their irregular shape and surface markings. They are stained by iodine. *Vegetable cells* are frequently seen. These have various shapes and all have a thick cell wall. *Pollen grains* may have the size of cysts; they are usually brownish in color and have distinctive surface



Pseudoparasites. Drawings made from actual specimens observed in iron-hematoxylin stained, Schaudinn fixed, fecal smears.

markings in various designs. Spherical clusters of minute spores of a fungus (*Sphaerita*) sometimes parasitize the trophozoites of the intestinal amebae.

Laboratory Technics

The laboratory procedure adopted should utilize the routine examination of fresh material, directly or after concentration; it should be remembered that trophozoites are lost when concentrations are employed. Most laboratories will be unable to use routine cultures. The procedure recommended for a clinical laboratory includes an initial examination of material in a wet smear, primarily as a screening technic to separate negative specimens from those that contain protozoa. If nothing resembling protozoans is found the specimen is discarded without further examination. Positive specimens may disclose some features which will identify the species, but it is often necessary to check by at least an iodine stain. For final and definite confirmation the permanent, fixed stain is employed for all stool specimens in which a presumptive diagnosis of intestinal protozoa has been made. *Most mistakes are due to failure to find rather than to failure to identify intestinal protozoa.*

Collection of Specimens. In general, the normally passed stools are quite satisfactory for examination. They are usually formed or mushy and, therefore, cyst-bearing rather than trophozoite-bearing. When the diagnosis can be made from cysts the need for "fresh" and postcatharsis stools and the inconvenience of collecting and handling such specimens are eliminated. Normal stools may be examined at the convenience of the laboratory some time during the day, cysts remaining practically unchanged for several hours; when examination must be postponed until the following day, specimens should be kept in the refrigerator to retard degenerative changes. When packaged so as to prevent dehydration, stool specimens may be shipped by mail and are satisfactory for examination if received within 48 hours. If longer periods of time may elapse the specimen should be preserved with Schaudinn's solution or 10 per cent formalin.

The important indication for *fresh* specimens is when the diagnosis must be made from living trophozoites: *Endamoeba histolytica* in dysenteric stools, in sputum, or in material obtained from a suspected abscess or by sigmoidoscopic examination of lesions of the bowel; *Trichomonas vaginalis* in vaginal or urethral discharges. Such material should be examined as soon as possible and the motility of the trophozoites is enhanced if the material is kept warm prior to and during the examination. Fresh stools may also be desired for identification of nonpathogenic trophozoites. Postcatharsis specimens may be necessary when the normal passage is hard and firm or when surveys are being made for all species; castor oil and liquid petrolatum should not be used as these substances appear in the feces as tiny refractile globules and interfere with the recognition of protozoans. The presence of barium also renders the specimen unsatisfactory.

Repeated Examinations. The trophozoites and cysts vary greatly in number; one day there may be hundreds in a single wet smear, and a few days later several smears may be examined before a single protozoan can be found. Regardless of the number present the chances of detecting the organisms depend mainly upon the skill and interest of the individual worker, and no definite rule can be made as to the number of examinations to be made on a single specimen or the frequency with which they should be repeated. At least two smears should be made of each stool specimen, one from the inside of the fecal mass and one from the surface. An experienced technician will find 75 per cent of infections by examining a single specimen and nearly all in a series of three stools, one

of which is mushy in consistency. The larger cysts and the motile organisms are most easily found, more errors developing in the detection of small cysts.

Wet Smears, Unstained. Unstained preparations of feces may be examined in physiological saline solution, saline-iron-hematoxylin solution, or in tap water. Other material and liquid feces may be examined directly or after dilution on the slide. This is a rapid and uncomplicated procedure, and the features of the living trophozoites and cysts are disclosed. While saline solution is usually used, saline-iron-hematoxylin (Lawless, 1946) presents some advantages. This solution tends to clump or coagulate fecal debris and produces a stained background. The trophozoites and cysts remain free in a clear field and their refractility, actually or by contrast, is increased; blue filters improve the picture. Compared with normal saline, the search requires less time and is less tiring to the eyes. The activity of the trophozoites is unchanged. The solution is made by adding 15 ml. of 0.5 per cent hematoxylin stain solution and 0.25 ml. of 4 per cent ferric ammonium sulfate to 75 ml. physiological saline.

Tap water is used to subject the parasites to the osmosis of a hypotonic solution. This serves three purposes: (1) to differentiate rounded-up trophozoites from cysts, the latter being unaffected; (2) to identify *Dientamoeba fragilis;* (3) to eliminate *Blastocystis hominis.*

Procedure. A drop of solution is placed on the glass slide. A small particle of the specimen is transferred to the slide with a toothpick or applicator and stirred in the drop of solution until a smooth emulsion is formed. The coverglass is applied, care being taken to avoid air bubbles: this may be accomplished by placing the coverglass perpendicular in the emulsion and moving it along the slide for about an inch, carrying the preparation with it; the coverglass, still perpendicular and touching the emulsion, is returned back to its original position and lowered to the surface of the slide as though it were hinged. The final preparation should be thin enough so that newsprint is legible through it.

In order to find and to identify the various species a special type of illumination is indispensable. The light from a good microscope lamp and the concave mirror are used; the substage condenser is lowered and the iris diaphragm adjusted until the objects in the smear have maximum refractility without glare. The protozoa can be detected with the 16-mm. objective as shining, refractile bodies usually colorless or with a faint greenish or bluish tint; low magnification is important to bring out refractility and to save time and cover more material. Suspected objects are studied with the 4-mm. objective for features which confirm them as protozoans or identify them as to species. The entire area of the coverglass should be examined as shown in the accompanying diagram. Negative findings with the low-power lens should be checked by an examination of several fields with the higher-power lens; small trophozoites and cysts may have been missed.

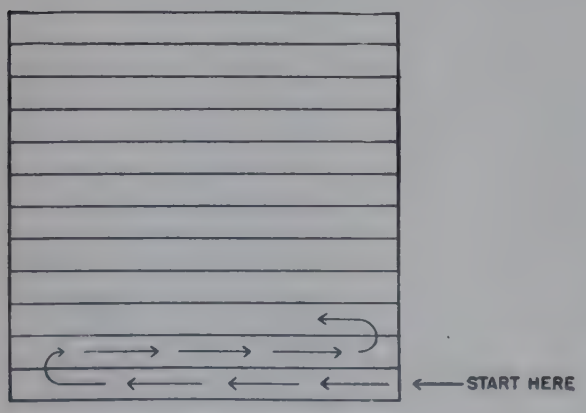


Diagram to illustrate the correct method of examining a fecal smear with a compound microscope.

Wet Smears, Iodine Stained. A wet smear is first made, preferably with tap water; this will eliminate the blastocysts which stain heavily; it will also destroy trophozoites, but iodine staining is not used for their identification. The smear is stirred with a toothpick or applicator dipped in Lugol's or D'Antoni's iodine solution until it is slightly tinted. When cysts are scarce, one is located in the aqueous smear with the low-power lens; it is kept in the field with the aid of the mechanical stage, and the iodine solution is introduced under the coverglass by capillary attraction.

LUGOL'S SOLUTION. Lugol's solution consists of the following:

Iodine crystals	5.0 Gm.
Potassium iodide	10.0 Gm.
Distilled water, q.s.	100.0 ml.

D'ANTONI'S IODINE SOLUTION. A 10 per cent potassium iodide solution is prepared by the specific-gravity method. From this reagent, a 1 per cent solution is prepared, to which 1.5 Gm. iodine crystals are added for each 100 ml.

Stained Fixed Smears. The recommended staining procedure uses Heidenhain's iron-alum hematoxylin. Either a short method or a long method may be used; the latter gives better definition and produces a more permanent stain. The rapid technic of Johnson (1935), or modifications, has an advantage in time saved, requiring about two hours or less. Staining dishes of 50- to 200-ml. capacity are usually used.

FIXING SOLUTION. A stock solution of Schaudinn's is prepared, two parts saturated solution of mercuric chloride and one part 95 per cent alcohol. From this, fresh fixative is prepared each day (or after every 10 or 40 slides) by adding glacial acetic acid to a strength of 5 per cent.

MORDANT. A 4 per cent solution of ferric ammonium sulfate is used as a mordant and a weaker solution of the same material as a decolorizing agent. Solutions must be prepared daily or after every 10 or 40 slides. All crystals used in making these solutions must be of a definite violet tint; white, yellow, or green crystals should not be used.

STAIN. A stock solution is made by dissolving 1.0 Gm. certified hematoxylin in 10 ml. of 95 per cent alcohol with the aid of heat; then 190 ml. distilled water are added. This solution must stand at room temperature in direct sunlight until "ripe," usually a few weeks, but this period may be shorter. The "ripe" stain has a reddish-brown color; a few drops added to a test tube of tap water gives a purple (not red) color. This test should be carried out on the used stain each morning, renewing it when it fails to give a satisfactory reaction.

PREPARATION OF SMEARS. Using a wooden applicator, a thin film of feces is spread on a glass slide; this is done quickly to prevent drying and then the slide is immediately immersed in the fixative. A drop of saline solution may be used to make a uniform smear from dry feces; in the absence of mucus, as in some liquid stools, a drop of egg albumen or serum will help the material adhere to the slide. The slides must be handled gently in the fixing solution to keep the smears from floating off. *At no time during the preparation, fixation, or staining can the smears be allowed to dry* as this causes shrinkage and distortion of the organisms.

STAINING, LONG METHOD:

1. Fix in Schaudinn's and acetic acid	60 min.
2. Dehydrate in alcohol, 50 per cent followed by 70 per cent, each	5 min.
3. Alcohol, 70 per cent with iodine to port-wine color	10 min.
4. Alcohol, 70 per cent, to remove iodine	10 min.
5. Alcohol, 95 per cent	5 min.
6. Hydrate in alcohols, 70, 50, and 30 per cent, each	5 min.
7. Distilled water	5 min.
8. Mordant in 4 per cent ferric ammonium sulfate	6 to 12 hr.
9. Rinse in three changes of distilled water.	
10. Stain with 0.5 per cent hematoxylin	12 to 24 hr.
11. Rinse in distilled water.	
12. Decolorize* in 1 per cent ferric ammonium sulfate	5 to 8 min.

*Decolorizing must be checked by removing the slides from time to time and placing them in water. A sample slide is taken from the water, the unsmeared side dried, and the wet film covered with a coverglass and examined with the microscope. At first the parasites will appear as fairly

13. Wash in running water ..... 20 min.
14. Dehydrate in graded alcohols, 50, 75, 95 per cent, and absolute, each ..... 5 min.
15. Clear in two changes of xylene ..... each 5 min.
16. Mount in balsam.

The time required for the procedure given above may be reduced in various ways: (1) by shortening the time in fixative, mordant and stain, with or without raising the temperature of these reagents; (2) by the addition of a detergent to increase the penetration of these reagents; (3) by the omission of some of the graded alcohols; (4) by the substitution of cellosolve for the final dehydration and clearing. Such methods produce a less permanent stain and distort the protozoa to some degree.

Staining, Short Method (Faust, 1945):

1. Fix in Schaudinn's solution at 60° C. ..... 2 min.
2. Alcohol, 70 per cent; alcohol, 70 per cent with iodine; alcohols 70 and 50 per cent, each ..... 2 min.
3. Wash in running water ..... 2 min.
4. Two per cent iron-alum at 40° C. ..... 2 min.
5. Wash in running water ..... 3 min.
6. Aqueous hematoxylin, 0.5 per cent ..... 2 min.
7. Wash in running water ..... 2 min.
8. Differentiate in cold aqueous iron-alum solution.
9. Wash in running water ..... 10–15 min.
10. Immerse in 70, 80, 90 per cent, and absolute alcohol, each ..... 2 min.
11. Clear with xylol.
12. Mount in xylol-balsam or clarite.

Concentration. Faust et al. (1938, 1939) have advocated a concentration method for detecting the cysts of the intestinal amebae. This procedure increases the total number of positives found and facilitates diagnosis when the organisms are scarce. It concentrates the ova of the common intestinal helminths as well as the cysts, reducing the usual search for intestinal parasites to one procedure. It is time-consuming.

1. One part of formed (cyst-bearing) stool is mixed with 10 parts of warm water in a glass container.
2. Ten ml. of this mixture are strained through one layer of wet cheesecloth in a glass funnel and into a suitable test tube.
3. This filtrate is centrifuged for 45 to 60 seconds at 2500 revolutions per minute.
4. The supernatant fluid is poured off; the sediment is resuspended in distilled water and centrifuged again. This is repeated until the supernatant fluid is clear.
5. To the final sediment are added 3 to 4 ml. of 33 per cent zinc sulfate solution (specific gravity 1.180). After the contents are thoroughly mixed the tube is filled to within one-half inch of the rim with the same solution.
6. This suspension is then centrifuged for at least 90 seconds.
7. Material from the surface of the liquid is transferred to a glass slide by means of a loop; a drop of dilute iodine solution is stirred into it; a coverglass is placed over the liquid and examination made for cysts.

Cultivation. This is of use in the detection of large-race *Endamoeba histolytica* and *Trichomonas vaginalis*. It gives poor results with small-race *E. histolytica* and is not successful with *Giardia lamblia*.

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solid black objects which become clearer as more stain is removed. If the color is not light enough, the coverglass is removed by a quick dip in the water and all the slides are returned to the ferric solution for further differentiation. This procedure must be repeated several times, usually at one-minute intervals, before the desired stain intensity is obtained.

Locke Egg-Serum Medium (Boeck and Drbohlav). Six eggs are washed with alcohol, broken, and emulsified in 75 ml. sterile Locke's (or Ringer's) solution. Four ml. are placed in test tubes and the tubes slanted in an inspissator and heated at 70° C. until the medium has solidified. The tubes are then autoclaved at 15 pounds pressure for 20 minutes. The slants are then covered with about 4 ml. sterile Locke's solution and serum (10 to 1) and a small amount of sterile rice starch added. After sterility tests, the cultures may be stored until used. This medium is suitable for cultivating parasitic amebae.

Complement Fixation. This test is of value in infections with *Endamoeba histolytica* when trophozoites or cysts cannot be demonstrated by repeated stool examinations. The technic, devised by Craig and modified by others, follows that of the Wassermann reaction. An alcoholic extract of cultures of *E. histolytica* is used as antigen.

Sigmoidoscopy. Some clinicians resort to sigmoidoscopy as a diagnostic procedure, hoping that there will be an ulcer within reach and that a smear from such ulcer will show the trophozoites of *Endamoeba histolytica.* This procedure is quite unnecessary since the parasites will be present in the feces, and abundantly so, in cases when the lesion is within such a short distance of the anus. Furthermore, failure to find ulcers and parasites with the sigmoidoscope swab has little significance since amebic lesions may be higher up in the colon, beyond the reach of the instrument.

CHAPTER 20

Medical Helminthology

The early systematists, Cuvier, Linnaeus, and others, included all wormlike animals that could not be placed elsewhere in a group called the *"Vermes."* It soon became apparent that this arrangement was an artificial one and that the Vermes consisted of three large phyla, more or less unrelated, and several distinct but smaller groups. The word "helminth," originally signifying "intestinal worm," has been broadened to include all parasitic forms of Vermes. The myriad of free-living species may also be called helminths when the word is used in its broadest sense. *Medical helminthology* deals with the parasitic worms of man. The importance of helminths to civilization is well emphasized by Stoll (1947) in an extremely interesting paper.

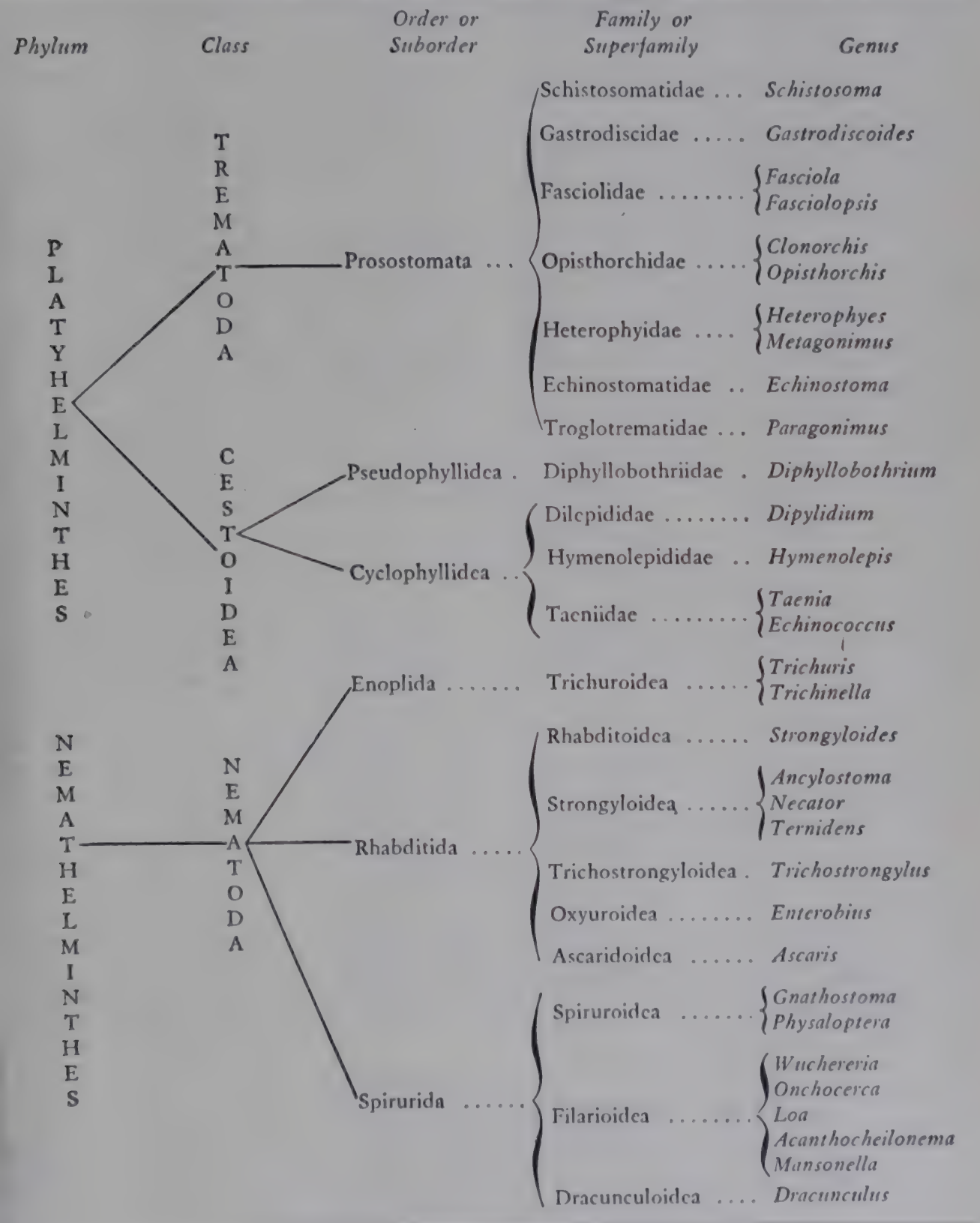
Classification. Most of the helminths of man belong to the phyla *Platyhelminthes* or flatworms and *Nemathelminthes* or roundworms. A third large phylum, *Annelida,* includes the segmented worms. The *Acanthocephala* or thorny-headed worms are more similar to the Platyhelminthes than to any other group; however, they are too distinct to be placed with the flatworms and most invertebrate zoologists consider them as a separate phylum. The *Nematomorpha* may be designated as a separate phylum or as a class of the phylum Nemathelminthes, to which they show, more or less, a superficial resemblance.

Morphology. Parasitic helminths have become greatly modified when compared with their closest free-living relatives. These changes, while often spoken of as degenerative, are actually specializations for parasitic life. Generally speaking, the most highly specialized or most greatly modified helminths of man are those which have had the most ancient association with him; tapeworms provide an excellent example. The site of location in the host is also closely related to the amount of modification which is necessary for parasitic existence; the parasites of the oral cavity are usually much more like free-living forms than those of the intestines; the tissue or somatic helminths, such as the blood flukes and the filariae, show great specialization.

The organs which are so vitally important to free-living forms—sense organs and those of locomotion and alimentation—are of little significance and, consequently, poorly developed in most parasitic forms. In contrast, organs of reproduction, attachment, and protection from the peculiar environment within a host are highly specialized. Cilia, very characteristic of the free-living Platyhelminthes, occur only in the free-living larval stages of the flukes and the more primitive tapeworms; suckers, the typical attachment organs of the flukes and tapeworms, are uncommon in the nonparasitic flatworms.

Table 60

CLASSIFICATION OF IMPORTANT GENERA OF HELMINTHS



All of the helminths parasitic in man are multicellular, bilaterally symmetrical animals having three germ layers. The various major groups present certain structural differences:

PHYLUM PLATYHELMINTHES. The gastrovascular cavity, when present, is usually without an anus; the excretory system is in the form of protonephridia with flame cells. These parasites are without body cavity; they are usually flattened, leaf- or tapelike.

CLASS CESTOIDEA. The alimentary canal is absent; the scolex is provided with suckers and often with hooks. The adults are nearly all flattened and tapelike. The parasite is hermaphroditic. Almost all members of this class have bodies made up of segments or proglottides. These parasites are known as *tapeworms* or *cestodes.*

CLASS TREMATODA. The alimentary canal is always present except in certain larval stages; suckers are almost always present. These parasites are nearly all flattened, leaflike; they are not segmented; they are known as *flukes* or *trematodes.*

PHYLUM ACANTHOCEPHALA. Members of this phylum are without an alimentary canal; an excretory system is present as protonephridia with flame cells in some. The parasites are without body cavity; they are usually more or less flattened when alive. The sexes are separate. These parasites are known as *thorny-headed worms.*

PHYLUM NEMATHELMINTHES. The alimentary tract is usually complete; the body cavity is not lined by an epithelium; the excretory system is without flame cells. The sexes are almost always separate. These parasites have no proboscides.

CLASS NEMATODA. Members have the characters of the phylum. They are known as *roundworms* or *threadworms.*

PHYLUM NEMATOMORPHA. Adults have a degenerate intestinal tract. The body cavity is entirely or partly lined by an epithelium. The excretory system is without flame cells.

CLASS GORDIACEA. The adults are free-living. The larvae are found typically in insects. The members of this class are accidental parasites of man; they are known as *hairworms* or *horsehair snakes.*

PHYLUM ANNELIDA. The alimentary tract is complete; a body cavity is present; the excretory system is without flame cells; the body is divided into a series of segments.

CLASS HIRUDINEA. These parasites have an anterior and a posterior sucker; they are hermaphroditic. Their bodies are flattened, leaflike. They are known as *leeches.*

Identification. All stages of diagnostic importance are differentiated in keys to be found in Chapter 24.

Helminths of Minor Importance to Man

Acanthocephala. These helminths are called thorny-headed worms because each possesses a proboscis which projects anteriorly like a little peg and is armed with several rows of hooks which are directed backward and enable the parasite to attach itself to the intestinal wall. The worm absorbs nourishment through the body wall, there being no mouth or alimentary canal. The sexes are separate. All these helminths are parasitic throughout their life cycles, usually having an arthropod intermediate host and a vertebrate definitive host.

Macracanthorhynchus hirudinaceus is normally an intestinal parasite of hogs. The male is 5 to 10 cm. long, the female 20 to 65 cm. The eggs, which are brown in color and about 100μ long, contain hooked embryos. The intermediate hosts are larvae of June bugs and related beetles. Human infestation is said to be common in southern Russia.

Moniliformis moniliformis, an intestinal parasite of rats, has been reported in man in a few instances. The male is about 4 cm. long, the female about 20 cm. The proboscis has 12 to 15 rows of hooks. A beetle, *Blaps mucronata,* and a cockroach, *Periplaneta americana,* are the intermediate hosts; possibly other insects are involved. Infestation may be contracted by eating deathwatch beetles, as is sometimes done to improve the complexion.

Nematomorpha. The hairworms or horsehair snakes belong to the class Gordiacea. They are long, threadlike forms with separate sexes and are parasitic in insects as larvae, the adults being free-living. The few cases of infestation in human beings probably result from ingestion of the adult in drinking water or of the larva within the insect host. These parasites may cause mild digestive disturbances.

Annelida. Leeches belong to the class Hirudinea of this phylum, members of which are parasitic and do not possess chaetae; they move about by means of a sucker at the posterior end. They have a rather oval body marked by numerous rings and a well-developed muscular system which enables them to contract and extend actively. There is also a sucker at the anterior end; within this is the mouth leading to the pharynx which serves as a pumping organ by the action of its muscular walls. The salivary glands, situated inside the mouth cavity, secrete the fluid which prevents coagulation of the blood. The mouth may or may not be provided with cutting jaws. In *Hirudo* there are three semicircular jaws, the arched surfaces of which are beset with from 50 to 100 sharp teeth; the mark of a leech bite is triangular. When a leech has gorged itself it becomes detached from the skin of its victim, but the effect of the salivary secretion on blood coagulation is of some duration so that the wound continues to bleed. Some wounds may become infected, and ulcers which may prove serious often result. This is particularly true with *Haemadipsa zeylanica,* a leech living on land but requiring abundant moisture; most species live in water. As a rule, leeches are hermaphroditic and reproduce by depositing so-called cocoons, rounded bodies surrounded by a shell and containing eggs in an albuminous matrix. Dimethylphthalate has recently been found to be a very effective repellent against land leeches (Ribbands, 1946).

Hirudo medicinalis is the leech used medically to induce bleeding. It is about 4 inches long and of a grayish-green color with dingy-red, longitudinal stripes on the dorsum and a dark-green ventral surface.

Limnatis nilotica is found in many parts of northern Africa, Palestine, and adjacent regions. The young leeches, only about 3 mm. long, gain access to the mouth in contaminated water. They attach themselves to the mucous membrane of the mouth, nose, larynx, or even trachea and remain there for several weeks until they reach adult size, up to 10 cm. long and 1.2 cm. wide. They may cause headache and obstinate bleeding, often resulting in severe and even fatal anemia. The dorsal surface of these leeches is greenish-brown with orange-brown borders.

Haemadipsa zeylanica is found in India, the Philippines, Australia, and South America. It leaves the damp earth to climb shrubs, from there dropping on animals or man passing through the forest. Its bite is painless but may be followed by an ulcer. This parasite may get into the nostrils and will even penetrate thick clothing to reach the skin.

CHAPTER 21

The Flukes or Trematodes

Nearly every vertebrate is the host of one or more species of flukes. Those forms which are parasitic in man also have, almost without exception, other available vertebrate hosts, often domestic animals. From this it might be expected that the flukes would tend to be cosmopolitan in distribution, as indeed they are in most lower animals; but in man they are found largely in the Tropics and the Orient. This is attributable, in part, to the food habits and the standards of sanitation in these areas, but other factors may be important. Since all of these helminths require a molluscan intermediate host, indigenous cases occur only where the suitable snail is present. However, the introduction of human cases into new regions in which a suitable intermediate host exists has resulted in the establishment of new endemic areas.

Classification. The parasitic flukes of man belong to the subclass *Digenea,* whose members are endoparasites with a complex development including an alternation of three or more generations (metagenesis) and an alternation of hosts; they also belong to the order *Prosostomata* which includes those forms with a mouth, surrounded by an oral sucker, located at the anterior end of the body. As a practical aid to study and identification, the trematodes of man are grouped for discussion according to their usual anatomic location in the human body.

Blood Flukes:

Schistosoma haematobium (Bilharz, 1852) Weinland, 1858
Schistosoma mansoni Sambon, 1907
Schistosoma japonicum Katsurada, 1904

Liver Flukes:

Fasciola hepatica Linnaeus, 1758
Clonorchis sinensis (Cobbold, 1875) Looss, 1907
Opisthorchis felineus (Rivolta, 1884) Blanchard, 1895
Opisthorchis viverrini (Poirier, 1886) Stiles and Hassall, 1896

Intestinal Flukes:

Fasciolopsis buski (Lankester, 1857) Odhner, 1902
Metagonimus yokogawai Katsurada, 1912
Heterophyes heterophyes (v. Siebold, 1852) Stiles and Hassall, 1900
Echinostoma ilocanum (Garrison, 1908) Odhner, 1911
Gastrodiscoides hominis (Lewis and McConnell, 1876) Leiper, 1913

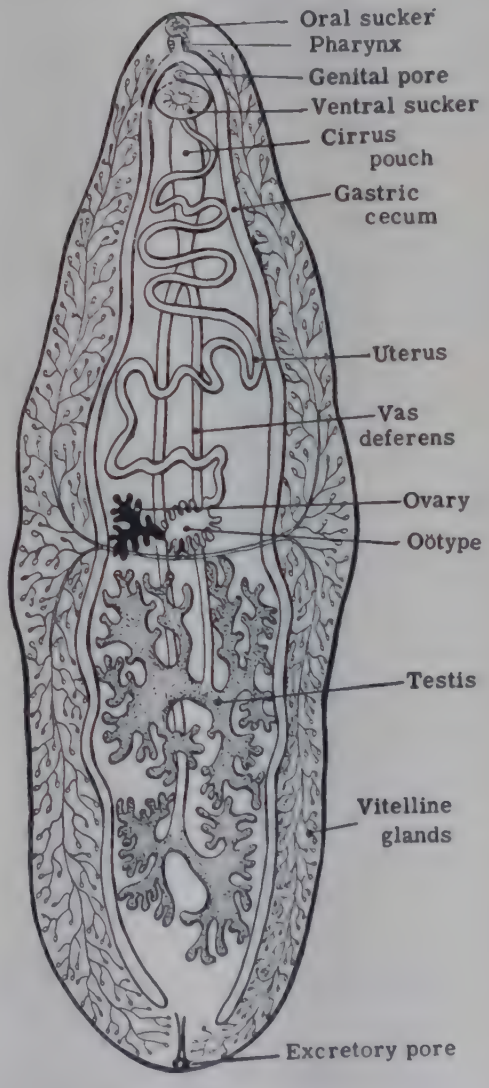
Lung Fluke:

Paragonimus westermani (Kerbert, 1878) Braun, 1899

In addition to these 13 species which have been found to occur in man in many cases, approximately 30 species which are rare, accidental, or spurious parasites of man are also known. If interested in these forms, the reader should consult a standard text in helminthology.

Morphology. Flukes are nonsegmented flatworms, usually leaflike in outline, rarely cylindrical. They are especially characterized by the possession of suckers by means of which they attach themselves to the skin, mucosa, or other tissues of the host. With the exception of the blood flukes, all human trematodes are hermaphroditic and their eggs are provided with a lid or operculum.

Adult flukes have two suckers which, except in *Gastrodiscoides,* are quite close together: an *oral sucker* and an *acetabulum*. The intestinal tract, proceeding from the mouth, usually consists of a pharynx, an esophagus, and two blind ceca; in the blood flukes the branches reunite to end in a single cecum and the pharynx is absent. There is no anus in any fluke that infects man.

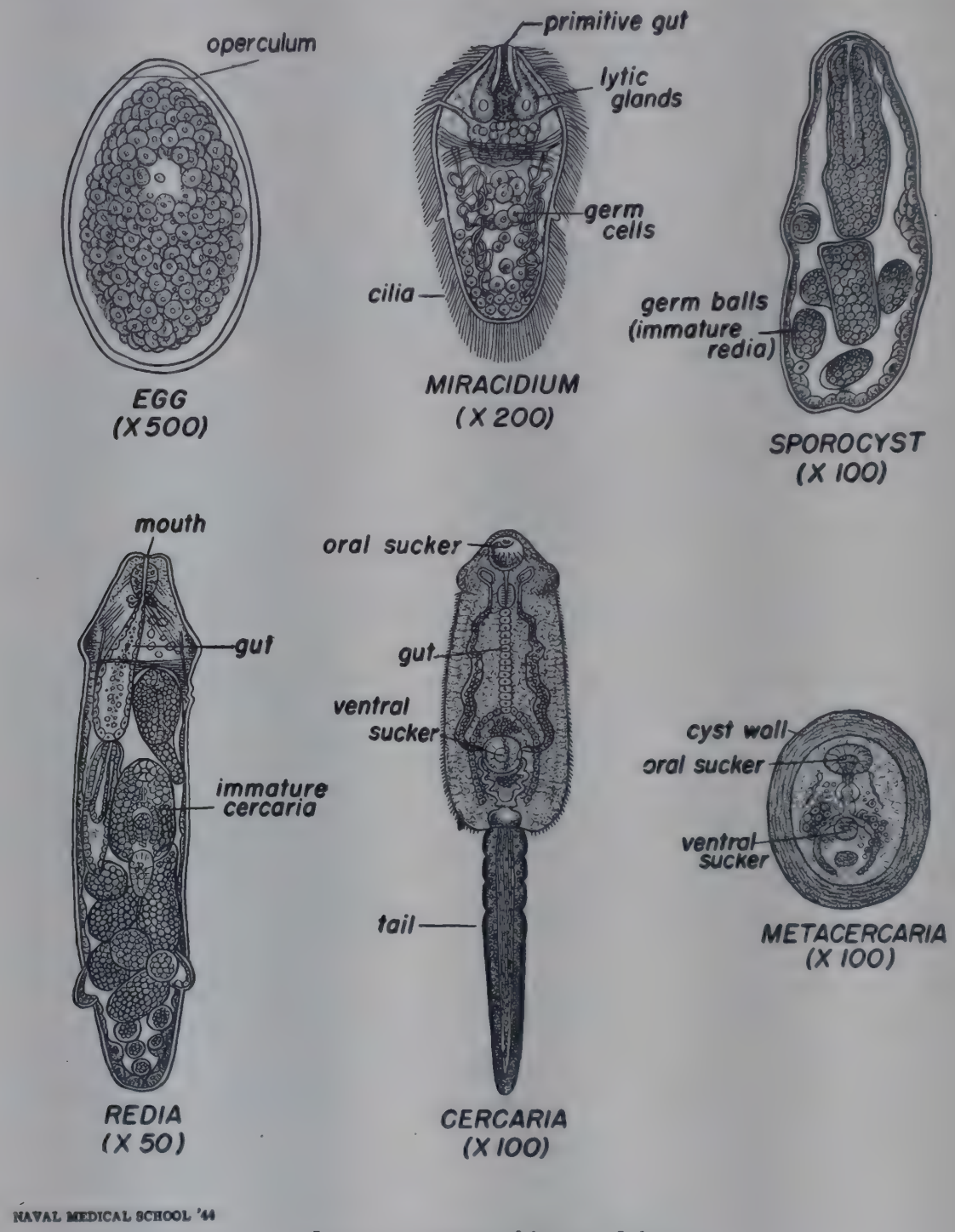


A fluke.

The excretory system consists of numerous, scattered *"flame" cells* from which minute canaliculi arise. These unite to form two collecting ducts which join posteriorly in the excretory vesicle, discharging through the excretory pore.

There are usually two *testes* which vary greatly in shape and position in different families. A *vas efferens* arises from each, and these unite to form the *vas deferens*. This discharges at the genital pore, usually situated ventrally, near the bifurcation of the gut in front of the ventral sucker. The terminal part is often modified to form a muscular copulatory organ, the *cirrus*. There is a single *ovary* from which the *oviduct* arises; this gives off a branch which usually ends blindly in a *receptaculum seminis,* but which may give off a secondary branch (Laurer's canal) which sometimes opens externally on the dorsal surface and is believed to take care of the overflow from the oviduct. The oviduct proper, in which fertilization of the ova takes place, receives ducts from the yolk glands and continues forward as the *uterus*. The uterus discharges the ova at the genital pore. The terminal part functions as a *vagina* at the beginning of sexual activity, but copulation appears to be impossible after the uterus becomes filled with ova.

IMMATURE STAGES OF HUMAN FLUKES



Immature stages of human flukes.

Ovum or Egg. An ovum or egg is a germ cell, or multicellular mass, surrounded by yolk cells and enclosed in a shell. Fluke eggs have either an *operculum*, a cap or lid which is forced off by the hatching embryo, or a characteristic *spine*. Although other helminths (primitive tapeworms) may have operculate ova, spined eggs are found only in the blood flukes (Schistosomatidae).

Miracidium. This may be defined as the ciliated larva which hatches from the egg. The majority of fluke eggs contain a well-developed miracidium when they are passed from the host. However, some require an incubation period. The developed miracidium has a short, saclike gut, several flame cells, and germ cells

or germ balls which are destined to give rise to a new generation of organisms.

Sporocyst. A sporocyst is a nonciliated form produced by the metamorphosis of the miracidium within the snail. This is an irregular, saclike structure containing germ balls which produce the next larval stage. There is no alimentary canal, food being absorbed through the body wall.

Daughter Sporocyst. This is a sporocyst arising from the germ balls of a primary mother sporocyst. The morphology is as described above.

Redia. This is a larval stage arising from the germ balls of a sporocyst. In a few instances, rediae may develop from the germ balls of a miracidium. Rediae are characterized by the possession of a mouth, muscular pharynx, and saclike cecum. Germ balls bud off from the wall of the redia and produce the next larval stage. A birth pore may be present to allow the passage of the succeeding stages at maturity.

Daughter Redia. A daughter redia is a redia developed within a primary mother redia.

Cercaria. A cercaria is a larva arising from the germ balls of either sporocyst or redia. It is characterized by oral and/or ventral suckers, a mouth, diverticulated gut, and tail. It leaves the tissues of the snail and swims about until it encysts on vegetation or finds and penetrates the next host. Cercariae with forked tails swim tail first; those with simple tails proceed with the body first.

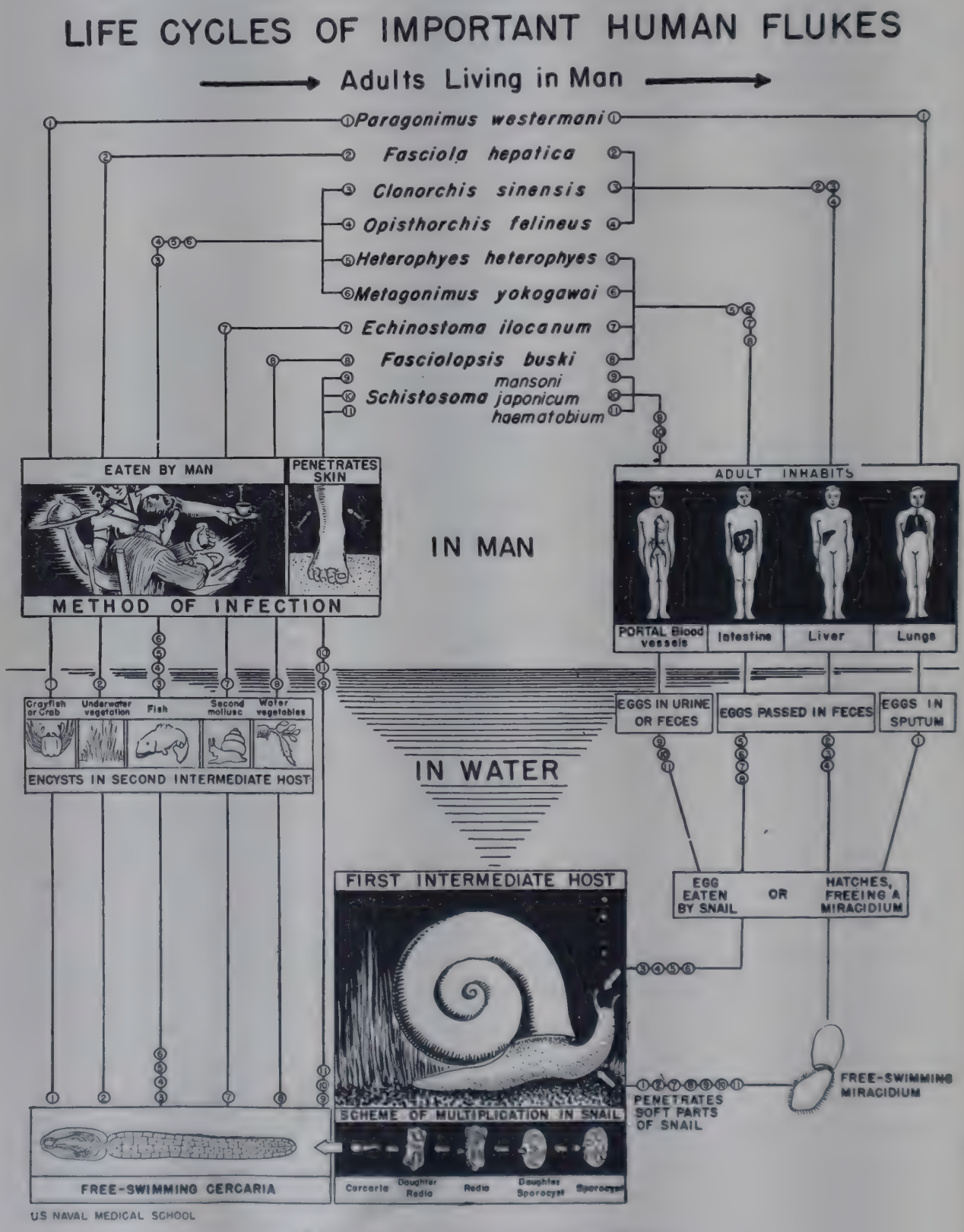
Metacercaria or Adolescaria. Metacercaria or adolescaria is a term applied to the stage between the cercaria and the adult fluke. This form is composed of the body of the cercaria, the tail being lost upon penetration of a second intermediate host or a definitive host. In species utilizing a second intermediate host, the metacercaria is an encysted form in this host and an adolescent developing fluke in the final or definitive host. The encysted form is absent in species which lack a second intermediate host (blood flukes), only the adolescaria developing.

Life Histories. The life histories of the important flukes infesting man are discussed collectively and depicted together on p. 564. By this means the similarities and differences are emphasized and, it is believed, an easier and more practical aid to learning and remembering is afforded. As listed under Classification, the adult flukes in man select various sites in the body. Eggs produced by these adults pass out in the urine, feces, or sputum, depending upon the location of the parasites. All ova except those of the schistosomes have a cap or operculum which opens to allow the miracidium to escape. Each species of trematode requires certain particular snails in which it can continue its development. The egg may hatch in water or it may be swallowed by the snail and hatch in its intestine. Regardless of where hatching takes place, the miracidium reaches the internal organs of the snail by penetrating the soft external tissues or the intestinal wall.

Upon gaining entrance into the tissues of the snail, the miracidium enters the intramolluscan period of its existence. It reproduces in large numbers during this time to compensate for the heavy mortality encountered during the passage from man (or other vertebrate) to the snail and to insure the survival of at least a few individuals during the passage from the snail to a definitive host or second inter-

mediate host. The miracidium loses its ciliated coat and becomes transformed into a second larval stage, usually a sporocyst but in certain cases a redia. The distinctions between sporocysts, rediae, and cercariae are purely morphologic. Regardless of the number of larval generations within the snail, the last stage produced is the cercaria. The cercariae leave the molluscan host and pursue various courses, depending upon the species involved.

The schistosome cercariae actively penetrate the skin of man by means of lytic



Life cycles of important human flukes.

glands; those of *Fasciola* and *Fasciolopsis* encyst on vegetation; those of *Clonorchis, Opisthorchis, Heterophyes,* and *Metagonimus* in fish; those of *Paragonimus* in crayfish and crabs; and those of *Echinostoma* in a second mollusk. The encysted metacercariae enter man when he eats these foods insufficiently cooked or raw. The metacercariae of the schistosomes, *Paragonimus* and *Fasciola,* undertake complicated migrations after entering man and before developing to sexual maturity; the other flukes undergo much less extensive movements.

Molluscan Hosts. In the trematodes of man of which we know the life histories, some genus of mollusk serves as an intermediate host. The phylum *Mollusca* includes unsegmented animals, usually contained in a calcareous shell and made up of a visceral mass, head, mantle, and foot. The first intermediate hosts of trematodes belong to the class *Gastropoda* which are known as snails and usually have a spirally coiled shell, a distinct head, and a broad, flat foot.

Experimental infection of snails by miracidia shows that these larvae tend to avoid unsuitable hosts and attack only certain species. Many miracidia, however, attack snails which are not efficient hosts and undergo only partial development. Various species of a genus of snails (but not all) may act as hosts. Mollusks are not so restricted in their range of trematode parasites or the flukes so restricted in their range of snail hosts as they were formerly thought to be. Methods of investigation and identification of snails are described in Chapter 24.

Prophylaxis. Prevention and control of human infections by the trematodes involve an understanding of the life histories and the epidemiology. Treatment of infected persons and proper disposal of human excreta are applicable for any of them. However, the situation becomes more complicated when other reservoir hosts exist in close association with man. Those infections acquired by ingesting the second intermediate host are most easily controlled by proper selection and preparation of food. Special measures must be taken to prevent infection by the cercariae of the blood flukes. These include complete avoidance of all infected waters. Every precaution should be taken against swimming, bathing, wading, or washing clothes in untreated water in endemic areas. Boiling the water is the most certain method of killing cercariae. When possible, water may be stored for at least 72 hours, the maximum period of cercarial survival; after routine treatment it may then be used for all purposes. Proper filtration and chlorination which leaves a residue of one part per million at the end of 30 minutes are also satisfactory.

Control measures are also directed against the snail intermediate hosts: removal of useless collections of water and vegetation; construction of irrigation canals and drains with cement; flooding with sea water, 35 to 40 per cent, for several days; and application of copper sulfate, copper carbonate, ammonium sulfate, or quick lime. Miller (1941) has found a mixture of copper sulfate, 2 parts, and copper carbonate, 1 part, dispersed on the bottom of a lake by a hose to be very effective for large water areas. Three pounds of the mixture will treat 1000 square feet. Adams (1945) recommends 5 parts copper sulfate (snow grade) and 1 part hydrated lime. Periodic drying of certain areas has not proved as effective as had

been hoped, but it will reduce the number of snails and their infectivity. Many of these measures are also effective against the free-living stages of the parasites. Steam applied to the mud has been used to kill snails in Japan; ducks have been introduced in some areas to feed upon snails. Recently (Naval Medical Research Institute, 1945) a combination of Phemerol, copper sulfate, and diesel oil has been found to be effective against snails, cercariae, and mosquito larvae. Buckley (1946) gives an excellent short summary of methods for the control of schistosomiasis.

The Blood Flukes

The most important flukes are those in which the adults are found in the blood vessels of man. Such infections are exceedingly common in Egypt and in certain areas of the Orient; they occur in many parts of tropical Africa and in South America. The disease is commonly called *schistosomiasis* as the parasites living in man all belong to a common genus, *Schistosoma*. It is also often referred to as bilharziasis from the important generic synonym, *Bilharzia*. *Schistosoma mansoni*, because of its wide distribution, high incidence and the presence of suitable intermediate hosts in the Americas, is the blood fluke of most importance to workers in the western hemisphere. The exposure of members of the armed forces to the oriental blood fluke in the Philippines, and possibly in China and Japan, has accentuated interest in *S. japonicum*. Although of wide distribution and high incidence in Africa, *S. haematobium* is probably of least importance to American students and technicians.

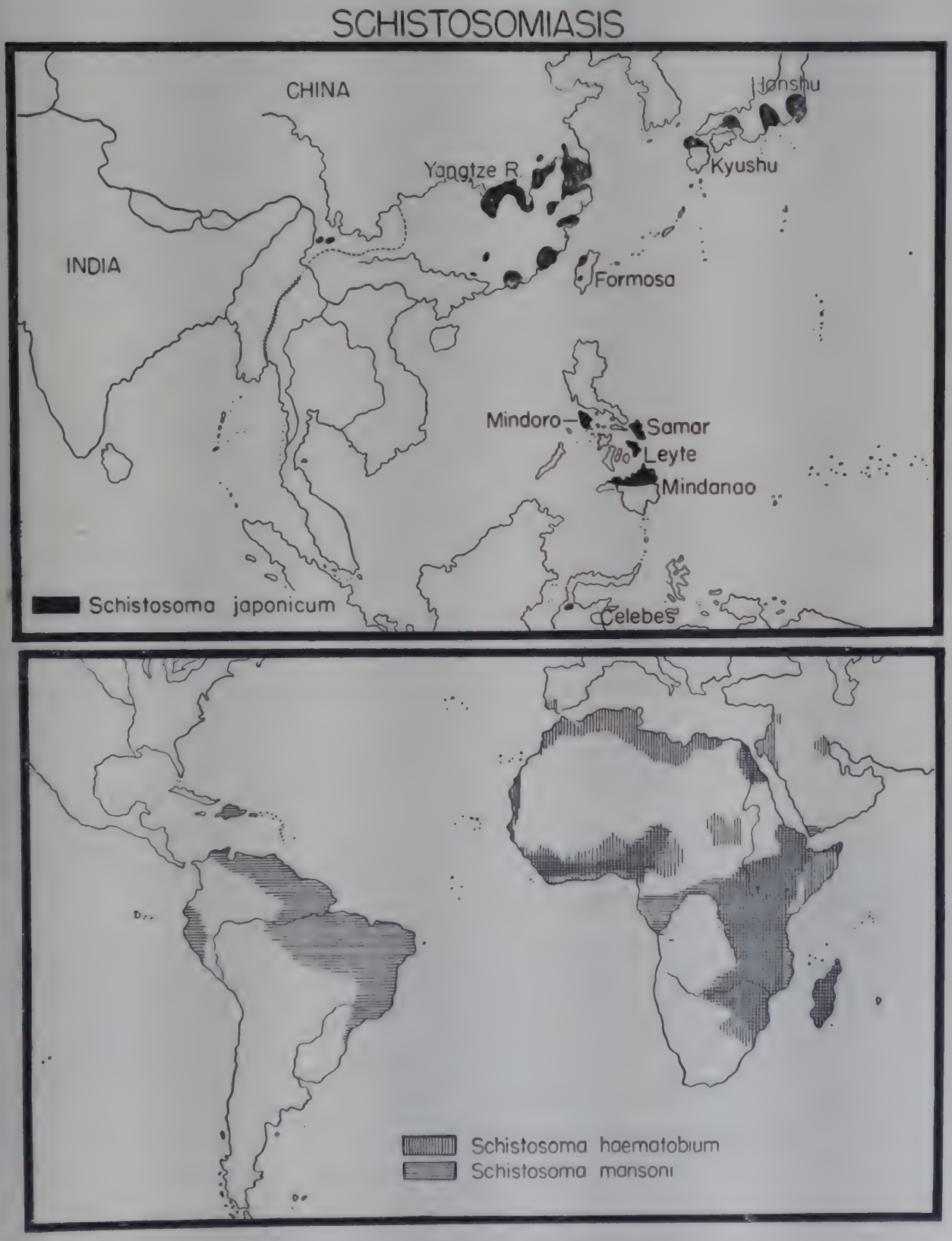
Species: Schistosoma Mansoni. *Schistosoma mansoni* is the cause of a chronic dysentery and of Egyptian splenomegaly. The disease may be referred to as schistosomal or bilharzial dysentery; schistosomiasis mansoni is preferable. In Africa, it occurs chiefly in Egypt and in the valleys of the Nile, the Congo, and the Niger rivers; it is also present along the northeast coast of South America, in the Amazon Valley, and in the West Indies, including Puerto Rico where it was presumably introduced by African slaves. In Venezuela, Scott (1940) found about 80 per cent of the males and 60 per cent of the females infected. The adult worms have a predilection for the mesenteric veins draining the bowel near the ileocecal junction. Their ova are deposited in the veins of the colon and especially those of the rectum, occasionally a few in those of the bladder.

Schistosoma Japonicum. *Schistosoma japonicum* also causes a chronic dysentery and great enlargement of the liver and spleen. Infection by this parasite has been called Katayama disease and oriental schistosomiasis but is preferably referred to as schistosomiasis japonica. It is common in certain districts of China, expecially the Yangtze Valley in which 10,000,000 people are said to be infected, and in southern Japan; it also occurs in the southern Philippines and the Celebes. The adults live in the superior mesenteric veins and discharge their eggs into the venules of the small bowel.

Schistosoma Haematobium. *Schistosoma haematobium* is the cause of endemic hematuria in Egypt where 60 to 70 per cent of the population are said to be infected. The disease is also called urinary or vesical schistosomiasis or schistosomiasis haematobia. The adult parasites live in the pelvic branches of the portal

system, and their eggs are deposited chiefly in the veins of the bladder, occasionally in those of the rectum. These flukes are long-lived and the infection may last 20 or 30 years. Eggs have been found in the lungs in about one-third of the cases at autopsy.

Morphology. The schistosomes differ from other flukes in many respects. The adults are dioecious (sexes separate) instead of hermaphroditic, there is no pharynx,



Geographic distribution of schistosomiasis.

and the gut branches reunite to form a single cecum. The eggs are spined and not operculate. In the larval stages, multiplication occurs only in the sporocysts, no rediae being formed. The cercariae have forked tails and no pharynx.

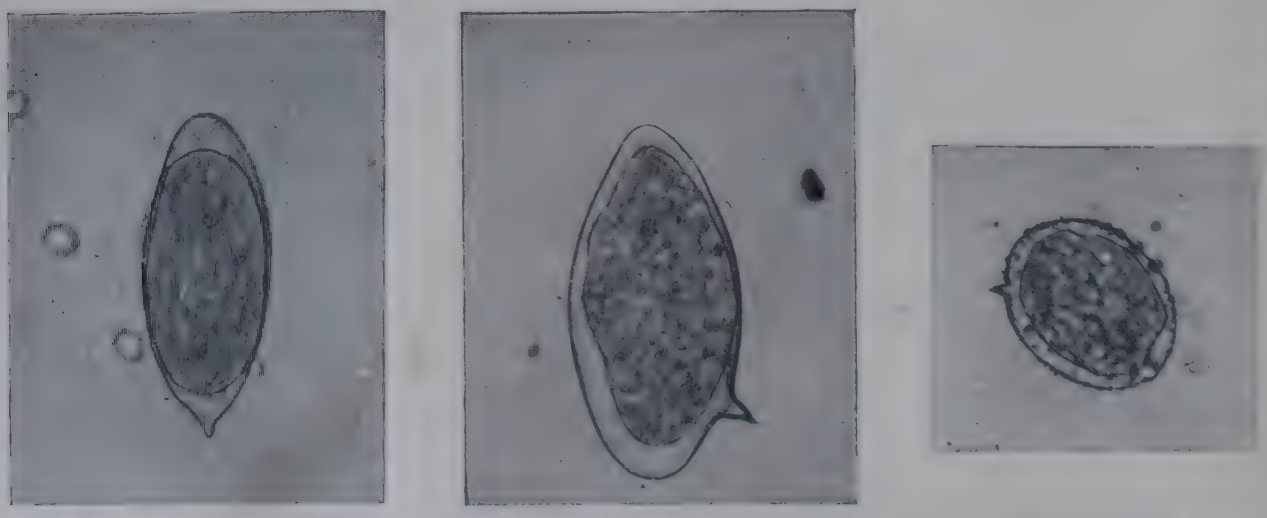
Table 61

DIFFERENTIAL FEATURES OF ADULT SCHISTOSOMES

	S. japonicum	*S. mansoni*	*S. haematobium*
Male			
Size (mm.)	9 – 22	6 – 14	10 – 18
Cuticle	Nontuberculate	Grossly tuberculate	Finely tuberculate
Testes	7	6 – 9	4
Reunion of intestinal ceca	In posterior fourth of body	In anterior half of body	About middle of body
Female			
Size (mm.)	12 – 26	12 – 16	15 – 20
Number of ova in uterus	50 or more	1 – 4	20 – 30
Position of ovary	Middle of body	Anterior body half	Posterior body half

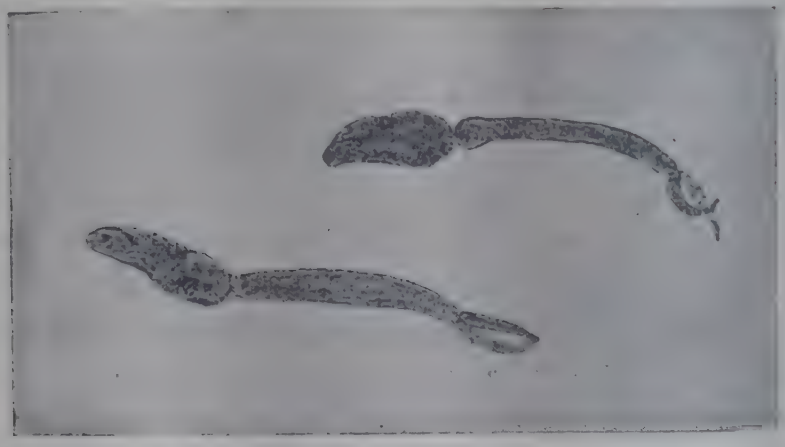
The anterior fifth of the adult male is cylindrical, but the remainder of the body is flattened and thin. The margins are infolded ventrally to form the *gynecophoric canal* in which the female is enclosed after sexual maturity is reached. The small testes are grouped just behind the ventral sucker. The female is long, cylindrical, and darker in color than the male; both extremities project from the gynecophoric canal in which she lives more or less permanently. The ovary lies anterior to the union of the gut branches.

Life Cycle and Transmission. The adult schistosomes live in the portal vein and its branches. According to the observations of Fairley and of Manson-Bahr on



Ova of three important human schistosomes. (*Left*) *Schistosoma haematobium*. (*Center*) *Schistosoma mansoni*. (*Right*) *Schistosoma japonicum*. (Photomicrographs × 200.)

living monkeys infected with *S. haematobium,* the paired worms travel against the blood stream into the finer branches of the mesenteric or pelvic veins. Here the female leaves the male and penetrates as far as possible into the venules, greatly distending them. As she withdraws, she deposits the ova, one at a time, with the spine pointing toward her. The elastic vein contracts down about the ovum, and the flow of blood tends to force the spine into the vessel wall. The ova cause a local inflammatory reaction and finally ulceration so that many of them reach the lumen of the bowel or bladder and are then passed in the urine or feces. The eggs, immature when deposited, contain a developed miracidium when passed. Schistosomes produce relatively few eggs at any one time as compared with other flukes. On reaching water the shell quickly ruptures, probably as a result of high osmotic pressure within the egg; the ova die within a few days in undiluted feces or urine. Upon its escape from the egg, the miracidium must find and penetrate a suitable snail host within a short time.



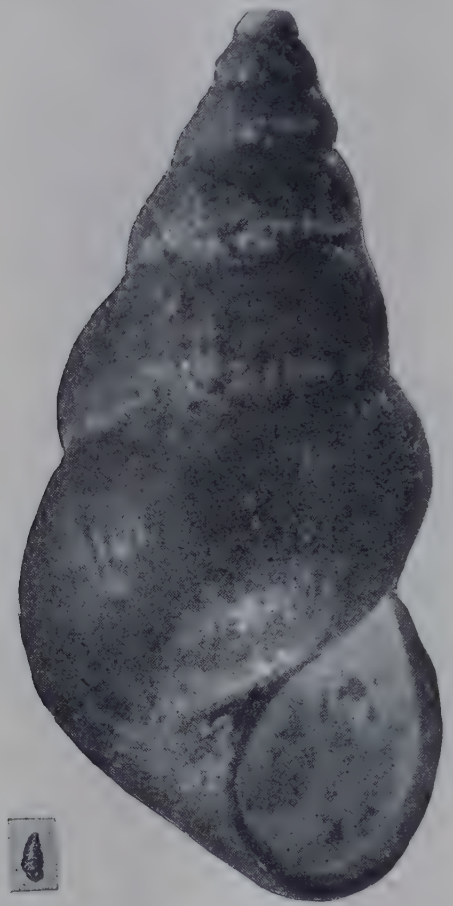
Cercariae of *Schistosoma haematobium.* (Photomicrographs × 100.)

A month or more is required for the development from miracidium to cercariae within the snail. The first generation sporocyst produces only a limited number of daughter sporocysts (25 to 50), but these latter second-generation larvae may produce numerous cercariae for long periods of time. Faust and Hoffman (1934) reported a snail infected by a single miracidium of *S. mansoni* discharged approximately 3500 cercariae each day.

Man usually acquires his infection by wading, bathing, or washing in infected water; if ingested in drinking water, the cercariae may penetrate the mucosa of the buccal cavity. Skin penetration occurs as the water evaporates and requires about 15 to 30 minutes. The metacercariae (adolescariae) are carried by the blood stream to the right side of the heart and thence to the lungs; after working their way through the capillary network, they return to the left side of the heart and pass out in the systemic circulation. Apparently only those which get into the mesenteric arteries (through the intestinal capillary bed) and into the liver (via the portal vessels) survive. The migration of *S. mansoni* from the skin to the lungs requires two or three days, and about four more to reach the liver. Once in the liver, the larvae grow rapidly into males and females and migrate down the portal vessels to the

venous plexuses in the wall of the intestine or bladder. The period required for ova to appear in the feces or urine varies from six to eight weeks or longer.

Man is the only important reservoir host of *S. mansoni* and *S. haematobium,* although monkeys have been found naturally infected. However, man is only one of a large number of definitive hosts for *S. japonicum;* Wu (1938) found sheep, goats, dogs, cats, house rats, oxen, buffaloes, and horses naturally infected in China. On Leyte, Magath and Mathiesen (1946) found pigs to be the most important reservoir host, dogs next, and rats of little significance.



Actual size Enlarged 17 ×

Snail intermediate host of *Schistosoma japonicum.* (Photograph.)

Numerous snail species may serve as the intermediate hosts for these schistosomes:

S. mansoni: Snails of the genera *Biomphalaria* and *Australorbis* are the usual hosts; *Physopsis* and *Bulinus* may be involved. Recent work indicates that related species in North America may also be suitable (Cram, Jones and Wright, 1945).

S. japonicum: Various species of *Oncomelania, Katayama,* and *Schistosomophora.* (The classification of these snails is uncertain at present. They may all belong to a single genus, *Oncomelania.*)

S. haematobium: Various species of *Bulinus, Physopsis,* and *Planorbarius.*

Clinical Illness. Occasionally the penetration of the skin by the cercariae is accompanied by a local dermatitis; it may be associated with intense pruritus and erythema, but this is uncommon. An incubation period of a month or more follows before the onset of symptoms and oviposition by the mature worms. Pains appear in the back, epigastric region, and legs or along nerve tracts; afternoon fever develops, often associated with loss of appetite, dry hacking cough, and general malaise. Frequently an intense urticaria with localized edema involving the subcutaneous tissue is associated with these symptoms. There is usually a leukocytosis and a marked eosinophilia, reported as high as 90 per cent.

Other symptoms are associated with lesions caused by the eggs of the parasites; they differ in detail with the species concerned. The wall of the bladder or intestine, in which most of the eggs are deposited, is primarily involved. However, many eggs are washed out of the veins and carried by the portal circulation to the liver and, in smaller numbers, to lungs, kidneys, and brain. They may even be found in the skin (Black, 1945). In some early cases on Leyte, cerebral symptoms were manifest (Carroll, 1946; Watson et al., 1947). Urinary frequency and hematuria are common in schistosomiasis haematobia; diarrhea may be marked in infections by *Schistosoma mansoni* and *S. japonicum.* Faust et al. (1946) give an

excellent discussion of the clinical picture of Oriental schistosomiasis which occurred in American soldiers on Leyte.

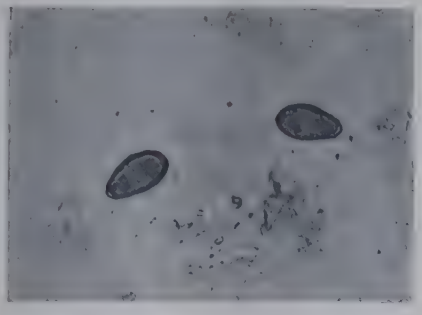
After a few years, progressive portal cirrhosis may develop. Chronic congestion causes enlargement of the spleen. Ascites frequently occurs. Other symptoms of chronic nature are caused by scarring and tissue hyperplasia or secondary infection. The patient may finally die of exhaustion or concurrent disease. As suitable treatment usually kills the adult parasite, it is evident that early diagnosis is of utmost importance. Recently, an intensive one-day treatment has been developed (Alves, 1946) which offers great promise for the control of schistosomiasis.

Cercarial Dermatitis (Swimmer's Itch). The cercariae of certain schistosomes normally parasitic in birds, muskrats, meadow mice, or cattle attack and penetrate the skin of man but are unable to develop into adults in this abnormal host. Intense itching of the infected areas develops several hours after exposure. This is accompanied by edema and the formation of a papular eruption which later becomes pustular, the reaction being most intense on the third day after exposure. Cercarial dermatitis occurs in the north, central and western portions of the United States and in Canada, England, France, Germany, Switzerland, New Zealand, and Malaya. If exposed to infection, immediate, vigorous wiping of the skin is advised.

The Liver Flukes

The liver flukes inhabit the bile passages, or occasionally the pancreatic ducts, which they reach by direct migration through the ampulla of Vater (*Clonorchis* and *Opisthorchis*) or by penetration of tissues (*Fasciola*). None are exclusively parasites of man, all being found in other mammals. Clonorchiasis is the only infection of major importance.

Species: Clonorchis Sinensis. The Chinese liver fluke is common in China, Indo-China, and Japan; in certain sections, over 75 per cent of the population are said to be infected. In addition to man, several other mammals, including the cat, dog, rat, and pig, serve as definitive hosts. Kobayashi has shown that the young flukes in cats reach the bile ducts 15 hours after infected fish has been ingested and require 26 days to reach maturity; they have been reported to live from 5 to 20 years. When squeezed out of the bile duct, the adults are so transparent and shiny as to resemble mucus; they are 10 to 25 mm. long and 2 to 5 mm. broad, pointed anteriorly. Thousands of these parasites have been found in a single person.



Ova of *Clonorchis sinensis*. (Photomicrograph × 200.)

The typical egg is shaped like an old-fashioned light bulb, is yellowish-brown in color, and contains a miracidium when passed. The operculum or cap fits into the thickened shell like the top of a sugar bowl with shoulders. A comma-shaped knob on the end opposite the operculum aids in identification; this is often difficult to see. Several types of abnormal eggs occur, one with an operculum but no embryo, a second without either lid or larva. The ova are very

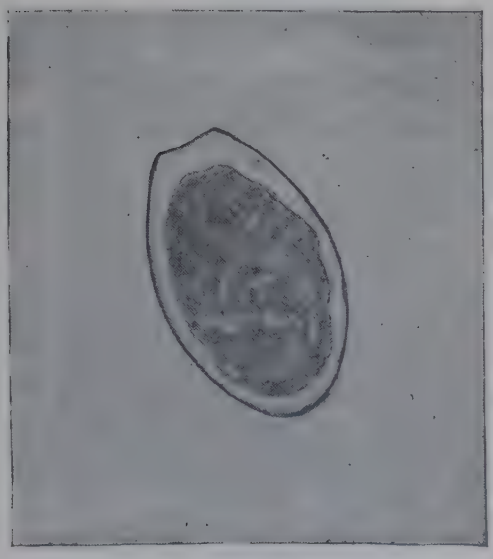
resistant and may remain viable as long as six months at low temperatures. The miracidium has an asymmetrical internal organization.

The cercariae which leave the snail host must find certain fresh-water fish within 24 to 48 hours or perish. Thorough cooking of the fish is essential for protection as the metacercariae can survive heating at 50° to 70° C. for 15 minutes.

Opisthorchis Felineus. The cat liver fluke is normally a parasite of cats, dogs, and pigs. It has been reported to be fairly common in man in eastern Germany, and in parts of Russia, Siberia, and Indo-China. The life history is similar to that of *Clonorchis,* the first intermediate host being a snail of the genus *Bulimus* and the second, various fresh-water cyprinoid fish. The yellow-brown ovum contains an asymmetrical miracidium when passed; compared with that of *Clonorchis,* the egg is more slender and has narrower and more tapering ends with less pronounced shoulders at the margins of the cap. The adult is about 10 mm. long × 2 mm. broad.

Opisthorchis Viverrini. *Opisthorchis viverrini* is normally a parasite of the civet cat. Stool examinations indicate it to be present in about one-fourth of the people in some parts of Siam. The ova resemble those of *Clonorchis,* and the life history is probably very similar.

Fasciola Hepatica. The sheep liver fluke, normally parasitic in sheep and other herbivorous animals, causes a destructive disease—liver rot. It has been reported in man in over 100 cases from the sheep-raising districts of the world. The adult is a fleshy, heart-shaped animal, measuring up to 30 × 13 mm., with relatively flat and slightly convoluted margins. There is a distinct conical projection at the anterior end, the posterior being broadly pointed. The two testes, which lie one behind the other in the second and third fourths of the body, are conspicuously branched.



Ovum of *Fasciola hepatica* (operculum detached). (Photomicrograph × 200.)

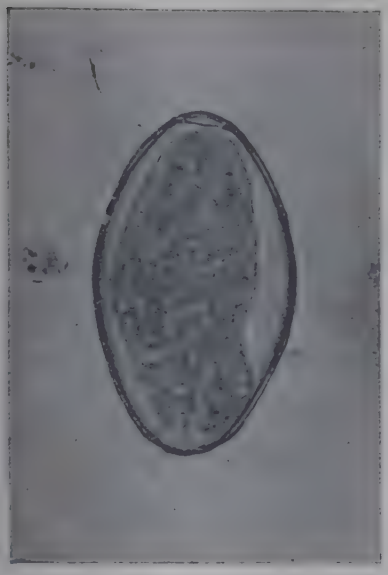
The life cycle is similar to that of *Fasciolopsis buski.* The ova are immature when passed and must develop to maturity in water; they remain viable up to nine months or longer. The miracidia hatch in the water and penetrate the tissues of an appropriate snail, species of no less than eight genera being susceptible to infection. Cercariae emerge from the snails late in the evening to settle and encyst on aquatic vegetation within eight hours. After excystation in the duodenum of the definitive host, the immature fluke penetrates the intestinal wall and migrates to the liver either directly or by the blood stream. Man appears to be as susceptible to infection as is the sheep. Eating uncooked watercress and other aquatic vegetation is the most likely method of infection. In the Gulf areas of the United States, Olsen (1947) recommends treatment of the infected sheep in the fall as the best method of control.

Clinical Illness. The clinical symptoms produced by liver flukes are usually proportional to the intensity of infection. In the presence of small numbers, only minor digestive disturbances may be manifest. Early heavy infections may also be mild or asymptomatic. In the more severe cases, diarrhea, enlargement of the liver, and jaundice develop, ultimately terminating with hepatic cirrhosis, ascites, and cachexia. Chronic cholecystitis, cholangitis, or pancreatitis may occur.

The Intestinal Flukes

Considering the severity of the diseases, infections by the trematodes which inhabit the intestinal tract are of less importance than those caused by liver flukes. With one exception, the species below are found in the small bowel where they become attached after excystation. All are parasites of domestic animals.

Species: Fasciolopsis Buski. The giant intestinal fluke is widely distributed in the Orient. It is the largest trematode found in man, measuring 40 to 75 × 12 mm., and is a common parasite of the pig in endemic areas. The adult is thick, brown in color, and has a very large acetabulum three to four times the size of the oral sucker and located adjacent to it. The branched ovary lies in the center of the body with the dichotomously branched testes posterior; the coiled uterus is anterior to the testes. This species is characterized by a very long, prominent cirrus.



Ovum of *Fasciolopsis buski*. (Photomicrograph × 200.)

The light yellowish-brown ova are immature when passed. Under favorable conditions they remain viable for six or more months. The cercariae live only a few hours after leaving the snail unless encysted on aquatic vegetation.

Metagonimus Yokogawai. This fluke is common in Japan, Korea, Formosa, Palestine, and the Balkans. It is found in cats, dogs, pigs, and some fish-eating birds as well as in man. The minute worm, 1 to 2 × 0.6 mm., has the acetabulum and the common genital pore displaced to one side. The light-yellow ova have thin shells with inconspicuous thickenings at the opercular margins; they contain miracidia with symmetrically arranged internal organs, mature when passed. Under favorable conditions the ova remain viable for months. The cercariae encyst under the scales of freshwater fish and reach the definitive host when this second intermediate host is eaten raw or insufficiently cooked.

Heterophyes Heterophyes. Normally a parasite of dogs and cats, this fluke occurs frequently, and often in large numbers, in man in Egypt, Palestine, China, and Japan. It is very small, 1.5 × 0.5 mm., and can be recognized by the large, prominent acetabulum; the oral sucker is much smaller. Very characteristic of the genus is the large, sucker-like genital pore just below and to one side of the acetabulum and surrounded by a collar of spines. The elliptic testes lie at the extreme posterior end. The cuticle has scalelike spines. The eggs are light brown, have a thick shell,

and contain a developed, symmetrical miracidium when passed; inconspicuous, thickened shoulders may be visible at the margins of the operculum. Both fresh- and salt-water fish have been incriminated as second intermediate hosts.

Africa et al. (1935) have reported finding several other species of heterophyids (apparently not well adapted to man as a host) in the wall of the intestines in nine out of 108 autopsies in Manila. No eggs were found in the feces and the intestinal lesions were trivial; however, many eggs, 10 to 16 × 25 to 33μ, were present in the myocardium in which they caused capillary embolism, areas of hemorrhage, and edema.

Echinostoma Ilocanum. *Echinostoma ilocanum* has been reported in man from the Philippines and the Celebes and in dogs and cats from other oriental regions. It is a small fluke 5 mm. long with two rings of spines around the anterior extremity—characteristic for the species. The thin-shelled eggs are inconspicuously operculate, oval, and immature when passed. Mollusks serve as both first and second intermediate hosts.

Gastrodiscoides Hominis. Normally a parasite of the pig, *Gastrodiscoides hominis* has been reported in man in India, China, and the Malay States. It inhabits the cecum and ascending colon. The adult, about 5 mm. long, consists of a posterior, concave, disclike portion from which proceeds a teatlike projection bearing an oral sucker; the acetabulum is in the posterior margin of the disc. The ovary is behind the testes. The greenish-brown, operculate ova are rhomboidal in shape, tapering toward each end, and immature when passed.

Clinical Illness. As with liver flukes, the symptoms due to intestinal trematodes will depend upon the intensity of the infection. Diarrhea may result from infection with any of the species. Complaints of abdominal pain or anorexia, nausea, and vomiting may be encountered. Some parasites may invade the mucosa and produce inflammation and ulceration. Heavy infections may result in death from cachexia or concurrent disease.

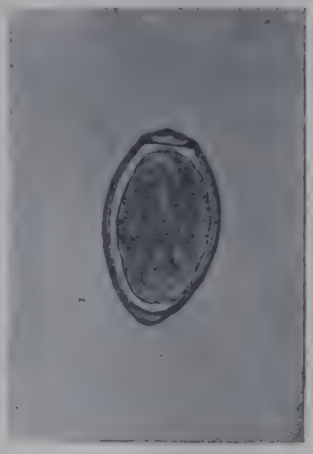
The Lung Fluke

The single lung fluke infecting man, *Paragonimus westermani,* has a very wide geographic distribution. It, or very closely related forms, has been frequently reported in parts of South America and in the Orient. Pigs, dogs, cats, and various wild carnivores as well as man are susceptible to infection. *Paragonimus kellicotti,* now generally regarded as a separate species, has been found widely distributed in North American mammals. Miller and Wilbur (1944) reported this parasite from several returning American servicemen.

The adult flukes are reddish-brown in color and rather fleshlike in appearance. They are from 8 to 12 mm. long, oval, and almost round on cross section with, however some flattening on the ventral surface. The acetabulum is conspicuous and lies anterior to the middle of the body on the ventral surface, just in front of the genital pore. The testes and ovary are branched; the ovary and the uterus are placed on opposite sides of the body, in front of the testes. The cuticle is covered with scalelike spines.

The golden-brown ovum has a thick shell. The operculum is definite with slight thickenings or shoulders at the margins. The eggs are immature when passed by the host and require several weeks under favorable conditions for development and hatching.

The second intermediate host is a crab (*Potamon, Eriocheir,* and others) or, in Korea, a crayfish in which the cercariae encyst. These crustaceans are often eaten raw or soaked in spiced wine in Japan and China; but in Korea and Formosa where the infection is also common, this custom does not prevail. Some believe that the encysted cercariae may enter the body in some other way. The young fluke which is liberated from the ingested cyst is believed to penetrate the intestinal wall, migrate through the peritoneal cavity, and burrow through the diaphragm into the lung. Here they are found, often in large numbers, in tunnel-like cavities or cysts lined by fibrous walls. They may live at least six years, possibly much longer.



Ovum of *Paragonimus westermani.* (Photomicrograph × 200.)

Clinical Illness. The prominent clinical symptoms resemble those of chronic bronchitis or bronchiectasis. The cysts in which the flukes live communicate with the bronchi. A chronic cough with abundant, rusty-brown, viscid sputum is usually present and hemorrhage may occur giving the disease the common name of "endemic hemoptysis." Pleural pain often causes trouble and lung abscess may develop. Other tissues may be invaded, including the liver, intestinal wall, testes, prostate, lymph nodes, skin, muscles, and brain. When located in such sites the flukes produce regional symptoms, notably a form of Jacksonian epilepsy when the brain is involved. Heavy infections lead to emaciation and loss of strength, and have a grave prognosis. The infection is very difficult to treat, the newer drugs having little or no effect (Brown and Hussey, 1947).

Diagnosis of Trematode Infections

The laboratory diagnosis of trematode infections depends almost entirely upon the finding and identification of the characteristic ova. All of the eggs may be passed in the feces, some of them being found exclusively in the contents of the intestinal tract. Of the others, ova of *Paragonimus westermani* normally appear in the sputum but are swallowed and found also in the feces in nearly one-half of the cases; eggs of *Schistosoma haematobium* are passed in the urine, rarely in the feces; and those of *S. mansoni* may very occasionally be found in the urine.

Feces should be examined by the direct smear, sedimentation, and centrifugation technics (see Chapter 24). *Flotation methods are not successful;* the ova of blood flukes become distorted, the large operculate eggs explode their caps and become unrecognizable, and the small operculate eggs are too heavy to be floated by the solution used. Hatching technics may be used for any of the ova containing a miracidium and have been found to give excellent results with the schistosomes. The acid-ether technic is also highly recommended for the eggs of *S. mansoni* and

S. japonicum (Weller and Dammin, 1945). Purgation may be a valuable aid; Hernandez Morales et al. (1945) found ova in 100 per cent of the cases of *S. mansoni* infection after such treatment, whereas only 58.3 per cent were positive before purgation.

In the examination of urine, essentially the same technics are employed as are used for feces; the last portion voided contains most of the eggs. Sputum is usually examined directly or after centrifugation; in paragonimiasis it contains minute iron-brown flecks formed by masses of ova and, usually, altered blood, eosinophils, and Charcot-Leyden crystals. The ova of liver flukes may be numerous in the bile when they are scarce in the feces. Eggs of *Fasciola* were recovered by duodenal intubation (Martin et al., 1944) 15 days before they appeared in feces.

Certain factors affect the occurrence of ova in the excreta. In early infections, before the adults are mature, obviously eggs will not be found. Unisexual infection by the blood flukes will not exhibit ova; such cases may not be rare, especially in individuals whose infection has resulted from a short exposure in the endemic area. Scarring and hyperplasia of tissues, as seen in the blood flukes, may prevent the ova from passing into the excreta; the number of eggs may be reduced by aging and death of the adults. Ova may occur in showers; Avery (personal communication, 1945) found that U. S. Navy personnel infected with *S. japonicum* on Leyte island would show ova in their stools in considerable numbers one day and then none for several weeks.

The great majority of ova can be distinguished by the characters given in Table 62; they may also be identified by the use of the key (see Chapter 24). Several cautions should be interjected: the eggs of *Fasciolopsis buski* and those of *Fasciola hepatica* are practically indistinguishable; *Clonorchis* and *Opisthorchis* ova are very similar, also those of *Heterophyes* and *Metagonimus;* they can be identified only after careful study. Finally, the operculate immature eggs of the pseudophyllidean tapeworms must not be confused with the somewhat similar ova of *Paragonimus* and *Echinostoma.*

Special tests are of value in schistosomiasis when the diagnosis cannot be confirmed by demonstrating ova; they may also be used as a criterion of cure. Complement-fixation, precipitin, and skin tests have been used by a number of investigators with excellent results; the skin test is perhaps the best aid as it combines ease of performance with accuracy (Culbertson and Rose, 1943; Gonzalez and Pratt, 1944). Williams (1947) found complement-fixation tests using cercarial antigen to be very useful in detecting chronic or persisting schistosomiasis. Intradermal and complement-fixation reactions with antigens from the adult worm have given excellent results in the diagnosis of fascioliasis (Lavier and Stefanopoulo, 1944). As euglobulin is increased in schistosomiasis, the aldehyde and similar tests may be used; other diseases which produce this change must be excluded. Recently Ottolina and Atencio (1943) found that biopsy of the rectal ampulla gave the highest incidence of infection with *S. mansoni;* biopsy of the liver gave the next and stool examinations the lowest rate of infection. Engelhardt (1942) has reported that a single provocative injection of 1.0 Gm. Bayer 205 would cause the

Table 62

DIFFERENTIAL CHARACTERS OF TREMATODE OVA

Species	*Size (in microns)*	*Special Features*
Schistosoma japonicum	70–105 x 55–80 Average 85 x 60	Nonoperculate; inconspicuous, lateral, curved spine, often not seen; contains miracidium
Schistosoma mansoni	110–180 x 45–75 Average 155 x 65	Nonoperculate; conspicuous *lateral* spine; contains miracidium
Schistosoma haematobium	110–170 x 40–75 Average 150 x 60	Nonoperculate; conspicuous *terminal* spine; contains miracidium
Clonorchis sinensis	27–35 x 12–20 Average 29 x 16	Distinctly operculate; contains asymmetrical miracidium; light-bulb-shaped; shoulders
Opisthorchis felineus	Average 30 x 12	Distinctly operculate; contains asymmetrical miracidium; shoulders not pronounced
Opisthorchis viverrini	Average 26 x 13	Distinctly operculate; contains asymmetrical miracidium
Fasciola hepatica	130–150 x 65–90 Average 140 x 80	Indistinctly operculate; immature; yolk granules concentrated around nuclei of cells
Fasciolopsis buski	130–140 x 80–90 Average 135 x 82	Indistinctly operculate; immature; yolk granules evenly distributed in yolk cells
Heterophyes heterophyes	28–30 x 15–17 Average 29 x 16	Distinctly operculate; contains symmetrical miracidium; shoulders not pronounced
Metagonimus yokogawai	26–28 x 15–17 Average 27 x 16	Distinctly operculate; contains symmetrical miracidium; shoulders not pronounced
Echinostoma ilocanum	85–115 x 55–70 Average 90 x 60	Indistinctly operculate; immature when passed
Gastrodiscoides hominis	150–170 x 60–70 Average 160 x 65	Distinctly operculate; immature; rhomboidal, tapering toward poles
Paragonimus westermani	75–120 x 45–65 Average 95 x 55	Distinctly operculate; golden-brown; immature; shoulders distinct but not pronounced

ova of *S. haematobium* to appear in the urine within 18 hours in latent cases. Proctoscopy (Faust, 1946) and sigmoidoscopy (Warren, 1947) have been found valuable in the diagnosis of *S. japonicum* infections in American servicemen.

Adult flukes may be recovered at autopsy, or intestinal flukes when passed in the feces after treatment, and identified by their characteristic structure (see key, Chapter 24).

CHAPTER 22

The Tapeworms or Cestodes

The tapeworms of man are almost all cosmopolitan in distribution and highly host specific. They cause severe disease in only exceptional circumstances, and as a rule the person harboring the parasites is unaware of their presence. These facts, in addition to the great specialization for parasitic existence as evidenced by the tapeworm's anatomy, indicate that the cestodes were probably parasites of ancestral man.

Classification. The class Cestoidea includes a very few primitive forms which are unsegmented, the subclass *Cestodaria,* and the very numerous segmented tapeworms, the *Cestoda;* all the forms found in man belong to the latter subclass. Only two orders of Cestoda contain parasites of man: The more primitive order, the *Pseudophyllidea,* contains tapeworms with a complex life cycle involving two intermediate hosts, a characteristic scolex with slitlike grooves, a uterine pore through which eggs are laid, operculate ova, and a ciliated larval stage. The other order, the *Cyclophyllidea,* contains forms with only one intermediate host or none, a scolex with four cup-shaped suckers, nonoperculate ova, and without a uterine pore or a ciliated larval stage. For convenience the various species found in man may be divided into two groups:

Intestinal Tapeworms—Adults living in the intestinal tract of man and other vertebrates; larvae in the tissues of vertebrates or arthropods.

Somatic Tapeworms—Larvae occasionally living in the tissues of man; adults in vertebrates. *Taenia solium,* normally belonging to the first group, may accidentally develop larvae in man and must be considered with both. The dwarf tapeworm which normally produces larvae in man is, for practical reasons, considered only with the intestinal tapeworms.

Intestinal Tapeworms:

Diphyllobothrium latum (Linnaeus, 1758) Lühe, 1910
Dipylidium caninum (Linnaeus, 1758) Railliet, 1892
Hymenolepis nana (v. Siebold, 1852) Blanchard, 1891
Hymenolepis diminuta (Rudolphi, 1819) Blanchard, 1891
Taenia saginata Goeze, 1782
Taenia solium Linnaeus, 1758

Somatic or Tissue Tapeworms:

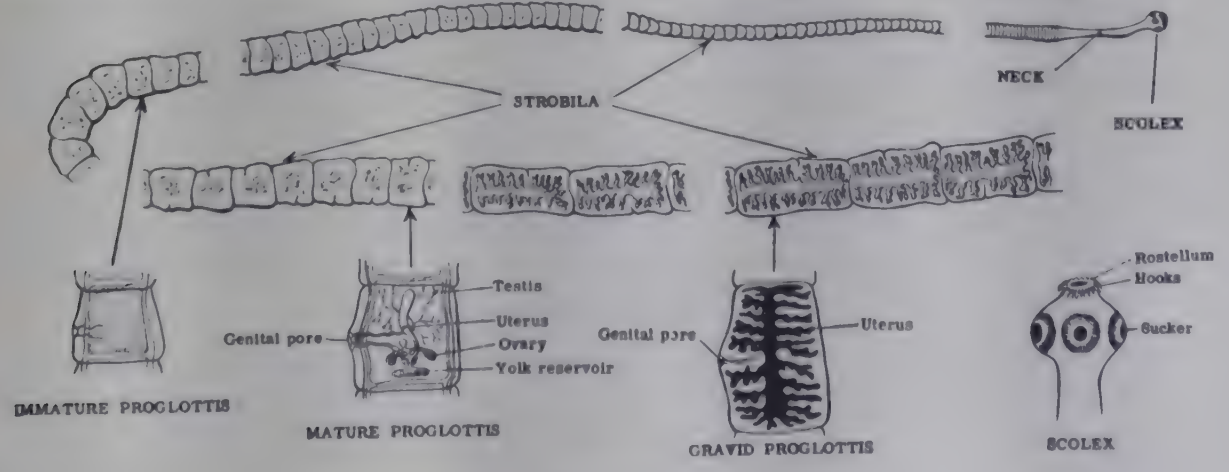
Echinococcus granulosus (Batsch, 1786) Rudolphi, 1805
Diphyllobothrium species
Taenia solium

In addition to these species which have been reported in many cases, there are approximately twenty others which are rare, accidental, or spurious parasites of man. A standard reference on helminthology should be consulted for information concerning them.

Morphology. *Adult tapeworms* are ribbon-like flatworms composed of a minute "head" or scolex, a neck, and usually a long series of segments or proglottides; with very few exceptions they are parasitic in the intestines of vertebrates. The *scolex* is more or less enlarged, globular or oval in outline, and structurally adapted for attachment to the intestinal wall. Immediately behind the scolex there is usually a slight constriction which is termed the *neck;* this is the budding zone composed largely of germinative tissue from which *proglottides* are formed. The segments are budded off at the posterior portion of the neck and, as they are pushed posteriorly by new proglottides, gradually develop. The proglottides may be grouped into regions according to their maturity; thus there is an *immature* region near the neck, a *mature* region near the middle, and a *gravid* region at the end, each region consisting of immature, mature, and gravid proglottides respectively. The scolex contains nervous tissue and the beginning of the water-vascular (excretory) system. There is no digestive system, nourishment being absorbed directly from the intestinal contents of the host.

Anatomically a tapeworm may be considered as a series of individual flukes united into a single tapelike colony. The proglottides may be regarded as sexually complete, hermaphroditic individuals, practically "egg factories." They are covered by an elastic cuticle and contain striated elliptical bodies composed of calcium carbonate. These *calcareous corpuscles* vary from 5 to 25μ in size and are characteristic of cestode tissue. Besides the sex organs, the proglottides contain a slender nerve fiber near each lateral margin and running the entire length of the worm; also a pair of excretory canals which usually communicate with each other by a transverse canal. The segments vary in number from three in *Echinococcus* to 3000 or more in *Diphyllobothrium latum.*

The reproductive systems closely resemble those of the flukes. Each proglottis contains a central *uterus,* often with a varying number of lateral branches, *ovaries* (near the ventral surface), and *vitelline glands* for the secretion of yolk. A *vagina*



A tapeworm.

leads from the *genital pore* to the *receptaculum seminis* and to the *oviduct* which is connected with the uterus in which eggs accumulate. There are also minute *testes* (from three to many in the tapeworms of man), a system of collection tubules, and a *vas deferens* opening at the genital pore and differentiated at its terminal end into an intromittent muscular organ, the *cirrus*. Ova may be fertilized by sperm from the same segment, from other segments of the same worm, or from other worms. The eggs are not discharged through the genital pore.

Two rather divergent types of reproductive systems are represented in the tapeworms. The first type is seen in *Diphyllobothrium* and its relatives; two genital openings are found on the surface of the segment, the male-female gonopore and the uterine pore; eggs are laid continuously by the gravid proglottis through the latter. The second type is found in all other tapeworms of man. A single (or sometimes double) genital pore is present at the side of the proglottis. The uterine pore has disappeared entirely and eggs cannot be laid; they remain in the uterus, dilating it into various patterns or forming little pockets, and the release of the ova is dependent upon the dissolution of the proglottis. This disintegration may occur in the intestine and then numerous eggs may be found in the feces; in other cases one or more gravid proglottides may detach themselves and be passed in the feces. In the case of *Taenia saginata* the segments may wriggle out of the anus or, if the feces have been deposited on the ground, may creep away into the grass and there disintegrate, liberating eggs where they are likely to be eaten by a cow.

Many of the *immature stages* are peculiar to the single species found in man; in such cases the description given applies to that species.

Egg or Ovum. An egg or ovum is a shelled structure produced by the mature proglottis and containing either a mature embryo or a zygote and numerous yolk cells. Two types of ova are encountered: the pseudophyllidean tapeworm has an operculate egg similar to that of certain flukes; the cyclophyllideans have nonoperculate ova in which three pairs of hooklets are visible.

Coracidium. The coracidium is the larva which hatches from the egg of the pseudophyllidean tapeworm. It is a ciliated, spherical, free-swimming form, usually about 50 to 55μ in diameter.

Onchosphere, Hexacanth Embryo or Six-hooked Embryo. This is the larva which hatches from the egg of the cyclophyllidean tapeworm; also that which remains after the disappearance of the ciliated coat of the coracidium. It is the fundamental embryonic form in all tapeworms; from it develop various stages in the different species.

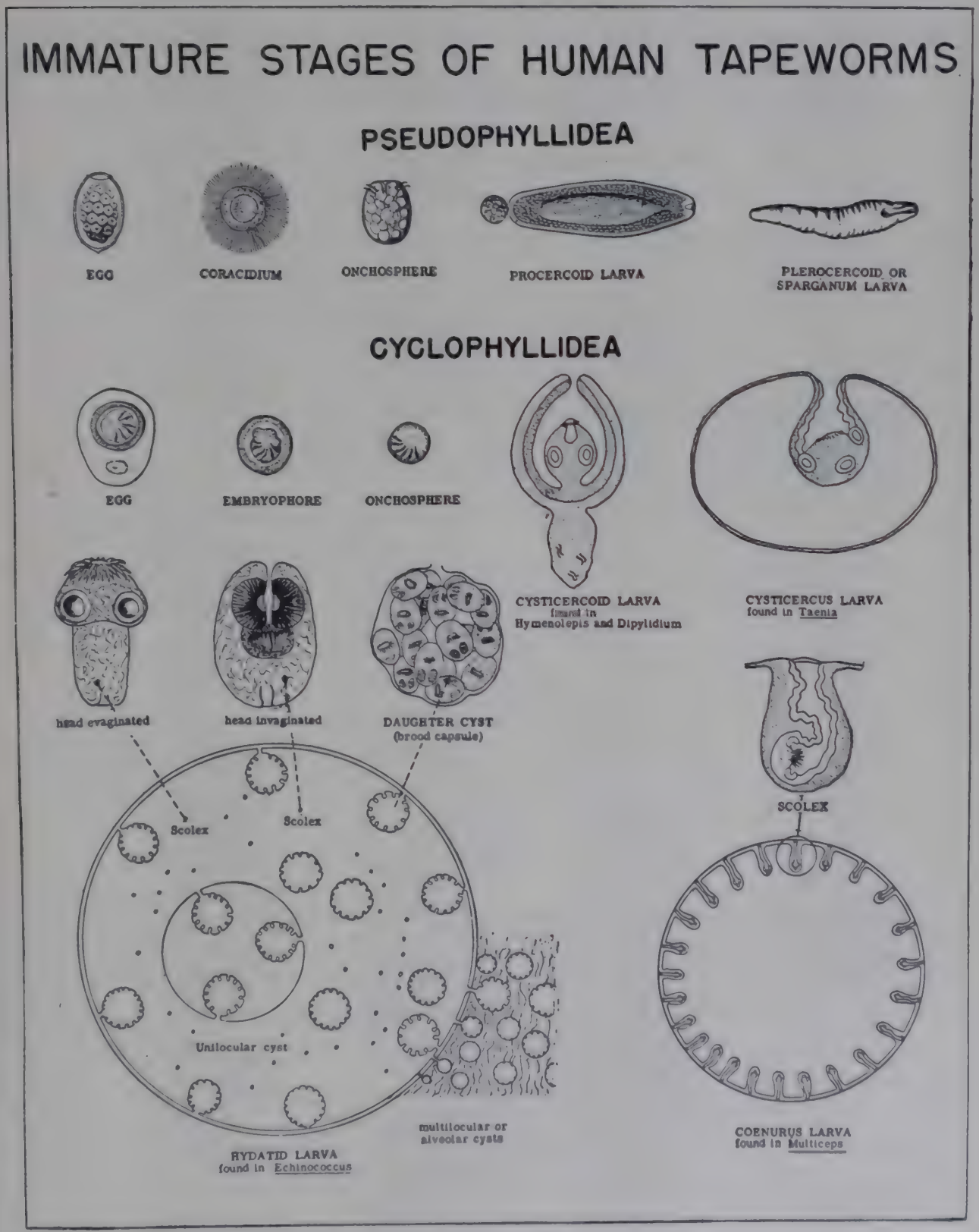
Embryophore. An embryophore is a cellular covering which surrounds the developing onchosphere of cyclophyllidean tapeworms. It is often erroneously called the egg shell, especially in *Taenia solium* and *T. saginata*. Some authorities consider the ciliated coat of the coracidium to be an embryophore.

Procercoid Larva. This is the larval form which develops from the onchosphere of the pseudophyllidean tapeworm. It is an ovoid or spindle-shaped organism about 50μ long with a posterior spherical protuberance (cercomer) which bears the six larval hooks.

Plerocercoid or Sparganum Larva. This is a larva developing from a procercoid. This form has lost the larval hooks and has developed a wormlike body with an anterior invagination. It is about 8 to 16 mm. long.

Cysticercoid Larva. This is the larva which develops from the onchosphere of *Hymenolepis* and *Dipylidium*. It has an invaginated scolex and a solid tail.

Cysticercus Larva. The cysticercus larva is that which develops from the on-



Immature stages of human tapeworms.

chosphere of *Taenia solium* and *T. saginata*. It has a single scolex invaginated into a single fluid-containing bladder; it is 6 to 12 mm. in diameter.

Coenurus Larva. The coenurus larva develops from the onchosphere of *Multiceps multiceps*. It is rarely found in man. It possesses many invaginated scolices within a single fluid-containing bladder.

Hydatid or Echinococcus Larva. This larva develops from the onchosphere of *Echinococcus granulosus*. It is characterized by having many invaginated scolices and many daughter bladders inside a mother bladder.

These last three larval stages form an interesting series showing increasing complexity. One adult tapeworm may develop from each cysticercus, hundreds from each coenurus, and thousands from each hydatid.

Intestinal Tapeworms

It can be expected that the cestodes which require only one host will be the most prevalent, those which utilize man and a domestic animal will be almost cosmopolitan, and finally those which need two intermediate hosts will be least common. Since most of the intestinal tapeworms of man fall into the first two categories, it may be said that tapeworms are found wherever man resides.

Morphology. Adult tapeworms differ greatly in their size and the number of proglottides; this is most easily presented in tabular form:

Table 63

Size and Number of Proglottides of Intestinal Tapeworms

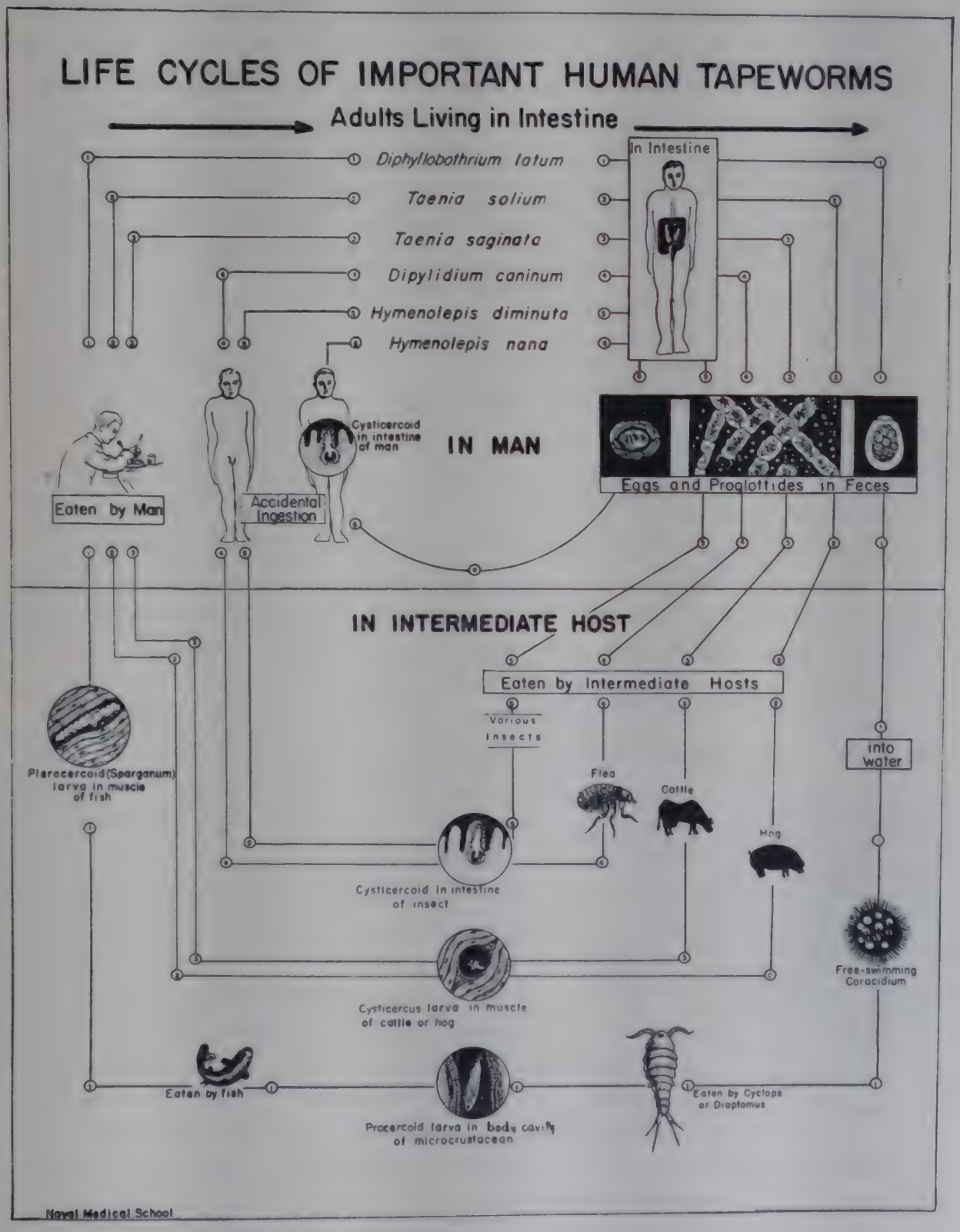
Species	*Size (in cm.)*	*No. of Proglottides*
Diphyllobothrium latum	300–1000	3000–4000
Taenia solium	200–700	800–1000
Taenia saginata	400–800 (occ. to 2500)	1000–2000
Hymenolepis nana	2.5–5.0	100–200
Hymenolepis diminuta	10–60	800–1000
Dipylidium caninum	15–70	60–175

Life Histories and Transmission. As was done with the flukes, the life histories of the cestodes are discussed together and outlined by diagram. The species of this group are presented in a series in which the outermost, *Diphyllobothrium latum,* shows a primitive type of life history involving one definitive and two intermediate hosts; and the innermost, *Hymenolepis nana,* exhibits a highly specialized adaptation requiring but a single host.

The ova of all tapeworms pass out with the feces, either after being laid (*Diphyllobothrium*) or within gravid proglottides which disintegrate before or after passage (*Taenia, Hymenolepis, Dipylidium*). When the ova of *Diphyllobothrium* are laid they are immature and develop to the infective stage upon reaching water; the ova of the other species listed are mature when passed—they contain a

mature onchosphere. These ova and the coracidium of *Diphyllobothrium* must be ingested by an appropriate intermediate host in which the larval stage develops. Adults develop in man or other definitive hosts when the larval stages are ingested with the tissues of an intermediate host.

Prophylaxis. Man becomes infected with intestinal tapeworms exclusively by ingesting infective stages. These may be the ova, as is the case in *Hymenolepis nana;* the larvae in food, as in *Diphyllobothrium latum* and the *Taenia* species; or



Life cycles of important human tapeworms.

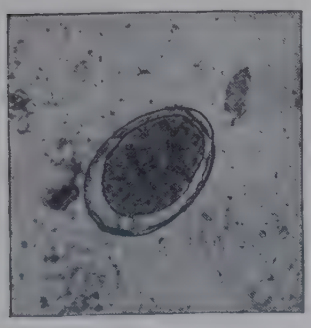
the larvae in arthropods, as in *Hymenolepis diminuta, Dipylidium caninum,* and others. Prophylaxis therefore consists primarily in controlling and treating food which may be infected and in avoiding conditions where accidental ingestion of infective ova or larvae is likely.

Those cestodes with larval stages in fish (*Diphyllobothrium latum*), beef (*Taenia saginata*), or pork (*Taenia solium*) may be avoided by thoroughly cooking the tissues used as food. Freezing, drying, pickling, smoking, and salting of fish have not been adequately studied and one cannot rely on these procedures at present. Prolonged refrigeration (−10° C. or lower for six days) or proper heating or pickling will destroy the larvae in beef or pork. Inspection of meat at the time of slaughter will disclose most infections, and the carcasses of beef or pork may then be appropriately handled. Treatment of infected persons and sanitary disposal of human feces is important for those species having only man as a definitive host.

In tapeworm infections acquired by ingestion of the infective larvae in arthropods the transmission is usually accidental. Children are more often infected because of their closer association with domestic pets. Control may be obtained by encouraging and enforcing personal habits of cleanliness, by simple limitation of pets, or, if this is not practical, by treating pets periodically to remove both the tapeworms and the insects acting as intermediate hosts. In *Hymenolepis nana* where the infective ova are ingested, habits of personal cleanliness are of utmost importance.

Species: Diphyllobothrium Latum. The broad fish tapeworm is the only important pseudophyllidean cestode found in man. It is common in the lake regions of Scandinavia, Russia, Switzerland, Bavaria, central Asia, Japan, and central Africa. It was introduced years ago into the region of the Great Lakes of North America and several cases have recently been reported from Florida. Dogs, cats, bears, and other fish-eating mammals serve as a reservoir of infection. Totterman (1944) believes that 14 per cent of all Finns are infected with *D. latum*.

The scolex is olive-shaped, 2.5 mm. in length, and has two deep suctorial grooves (bothria) on each side; rostellum and hooklets are absent. The uterus is rosette-shaped; separate uterine and genital pores are located on the ventral surface. Each gravid segment periodically discharges eggs in large numbers in the feces, up to 1,000,000 daily. The eggs are operculate, golden-yellow in color, with a thin shell enclosing a central mass of granular, spherical segments.



Ovum of *Diphyllobothrium latum*. (Photomicrograph × 200.)

When an egg reaches fresh water, segmentation proceeds if the temperature is favorable, and after several weeks the operculum opens to release the ciliated coracidium. The latter can live for several days in water, swimming about until swallowed by a microcrustacean (*Cyclops* or *Diaptomus* sp.), the first intermediate host. The coracidium loses its ciliated covering, pierces the gut wall, and develops in the tissues as a procercoid larva. When the crustacean is eaten by certain fresh-water fish (pike,

pickerel, perch, trout, salmon), the second intermediate host, the larva penetrates the stomach wall and passes into the muscles or other tissues where it develops into the plerocercoid or sparganum larva. Large carnivorous fish may also acquire this stage indirectly by ingesting smaller infected fish; sparganum larvae are able to reinvade and live in host after host. When viable larvae in the fish are ingested by man or other suitable definitive hosts, they are liberated in the intestine and develop rapidly into adults. In five or six weeks one may reach a length of 2 feet and begin to discharge eggs; it may live for years.

Taenia Saginata and T. Solium. The beef and pork tapeworms are found throughout the world wherever beef and pork are eaten. Next to *Hymenolepis nana,* the beef tapeworm (*Taenia saginata*) is the most common cestode of man. The two taenia species are very similar morphologically; differentiation is made primarily on the gravid proglottides, secondarily on the scolices, and rarely on the mature segments.

Distinguishing Features of T. saginata and T. solium

	Taenia saginata	*Taenia solium*
Scolex:		
Shape	Pearlike	Globular
Size (in mm.)	1 to 2	1
Rostellum	Absent	Present
Hooks	None	Double row
Mature proglottides:		
Testes	About 150	About 300
Ovary	Bilobed	Trilobed
Gravid proglottides:		
No. of main uterine branches	Range 15–30 Average 20	Range 5–13 Average 9

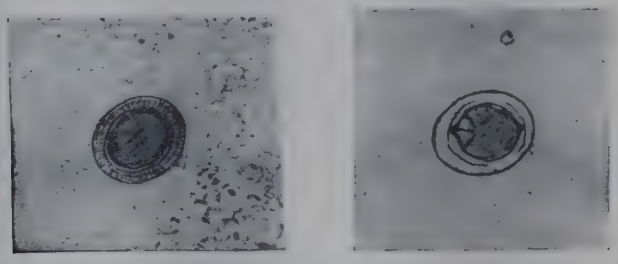
The eggs are very similar and the species cannot be distinguished by them; those of *T. saginata* may be slightly more ovoid. They possess a thin, transparent outer shell which is usually lost when the segment disintegrates. Within the outer shell there is a thick, porous, radially striate layer, the embryophore, containing the embryo proper (the onchosphere) with three pairs of hooklets.

The life cycles are very similar except for the intermediate hosts. Only two hosts are ever involved, man and bovines or man and hogs. After ingestion by the cow or pig, the onchospheres are liberated and penetrate the intestinal wall, migrating by way of the blood and lymphatics to certain tissues, expecially the diaphragm, heart, tongue, and other organs where they become encysted as cysticerci. The larvae of *Taenia saginata* are usually smaller than those of *T. solium;* man may serve as the intermediate host of the latter, but all reports of the larval stage of *T. saginata* occurring in man are open to question. The absence of hooklets on the scolex of *Cysticercus bovis* distinguishes this larva from that of the pork tapeworm, *Cysticercus cellulosae.* Development requires about three

months, and the encysted stage may remain viable for a long time (for years in *T. solium*). When man ingests infected beef or pork in which the larva has not been killed, the cyst wall is digested away and the scolex attaches itself to the intestine. The larva grows rapidly, reaching the adult stage after two months. The maximum duration of adult life is not known but is at least several years.

Hymenolepis Nana. The dwarf tapeworm is the smallest of the cestodes found in man and is the most common species in the United States. It has been estimated that 10 per cent of the children in parts of Italy are infected. This helminth is probably identical with a common rat tapeworm, *H. nana* var. *fraterna.*

The adult worms live in the upper ileum and are often numerous—1000 to 1500 or more. There is a lateral genital pore, on the same side of all segments. The head (0.3 mm.) has four suckers and a retractile rostellum encircled by a single row of 20 to 30 hooklets. The ova are liberated by disintegration of the terminal segments of the adult worm. They are oval, colorless, and translucent with a thin outer shell and an inner membrane leaving a zone about 7μ wide

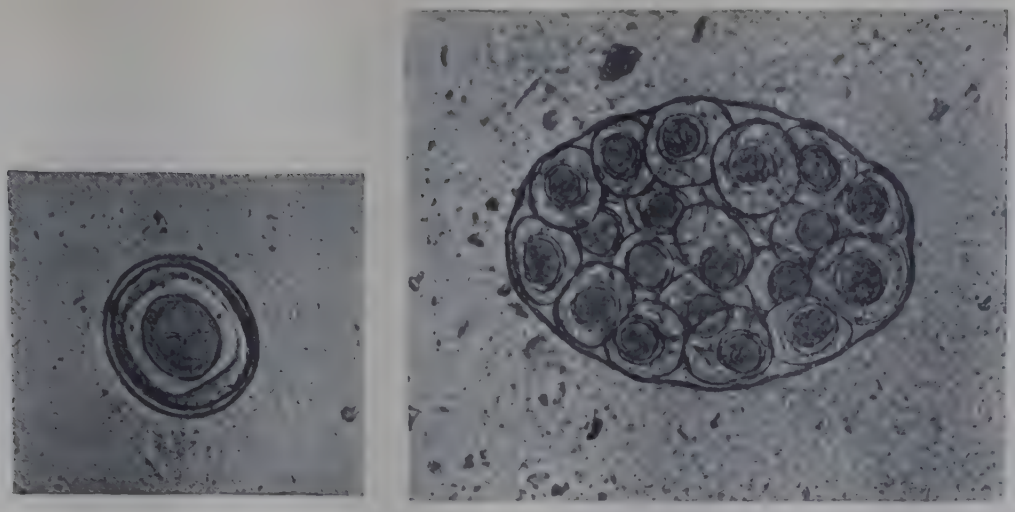


(*Left*) Ovum of *Taenia.* (*Right*) Ovum of *Hymenolepis nana.* (Photomicrographs × 200.)

between them. At each pole of the inner shell there is a slight protuberance from which arise four to eight long filaments; these lie in the zone between the two membranes and partly encircle the embryo. The latter contains six conspicuous hooklets.

Unlike other cestodes of man, this parasite requires no intermediate host. In the rat, and probably in man, the embryo is liberated from the ingested egg in the small intestine and penetrates a villus in which it encysts as a cysticercoid larva. A rostellum with hooklets appears after four days' development. The larva then leaves the villus, re-enters the lumen of the bowel, and attaches itself to the mucosa. Development is rapid so that eggs appear in the feces after about one month. They are infective as passed, and it is therefore easy for the host to become superinfected if feces are conveyed to the mouth by dirty fingers. Contamination of food by feces of infected rats is probably the usual source of infection. It is also possible for hyperinfection to take place; in this case the eggs liberated in the intestine immediately hatch and their onchospheres penetrate the intestinal wall. This phenomenon has been observed in mice.

Hymenolepis Diminuta. This flavo-punctate tapeworm of mice and rats has been found many times in man, chiefly in children. The scolex carries small suckers and a rostellum without hooklets. The ova resemble those of *H. nana* but are larger, have a thicker, radially striated outer membrane, and no filaments. The



(*Left*) Ovum of *Hymenolepis diminuta*. (*Right*) Ova of *Dipylidium caninum*. (Photomicrographs × 200.)

intermediate hosts are various species of insects and myriapods, including the rat flea whose larvae ingest the eggs. Man and rodents acquire the parasite by swallowing the infected arthropod.

Dipylidium Caninum. This is a common tapeworm of dogs and cats and has been occasionally found in man, chiefly in children. The head has four suckers and a rostellum with usually three or four rows of encircling hooklets. The eggs are similar to those of *Hymenolepis nana* but are grouped in packets of 5 to 25. The gravid proglottides often do not disintegrate until after passage in the feces. The parasite is acquired by swallowing infected fleas or lice, the intermediate hosts. The flea is infected in its larval stage.

Clinical Illness. The presence of cestodes in the intestinal tract of man is usually accompanied by rare or minor symptoms. Heavy infections may affect the nutrition of the host by absorption of food or interference with digestion. The well-known macrocytic anemia seen in diphyllobothriasis is a very rare complication, about two or four cases per thousand in Finland.

Diagnosis. The laboratory diagnosis of the intestinal tapeworms is made by finding and identifying the characteristic ova or proglottides in the feces; occasionally scolices may be recovered and identified. The only certain criterion of cure is the finding of the scolex of the worm; if the head is not removed the worm may regenerate. The hunt is often laborious and difficult. Methods of finding the ova or embryophores should include the direct smear, sedimentation, and centrifugation (see Chapter 24). Flotation technics are not satisfactory for the operculate ova of *Diphyllobothrium* or for the embryophores of *Taenia saginata* and *T. solium;* however, they give excellent results with *Hymenolepis nana* and *H. diminuta*. Russian workers and others have recently shown a substantial increase in positive diagnoses of *Taenia saginata* by scraping the perianal skin. The NIH swab and the scotch-tape technic should both be applicable; such methods may be of value for *T. solium*.

Where gravid proglottides are passed unbroken in the feces (*Dipylidium, Taenia*) screening methods must be used. A saline purge will usually cause some

Table 64

DIFFERENTIAL CHARACTERS OF CESTODE OVA

Species	*Size (in microns)*	*Special Features*
Diphyllobothrium latum	55–75 × 40–55 Average 70 × 45	Indistinctly operculate with knoblike thickening on abopercular end; immature
Taenia solium	30–40 (diameter) Average 35	Nonoperculate; embryo with hooks; thick, brown, radially striated embryophore
Taenia saginata	30–40 × 20–30 Average 35 × 25	Nonoperculate; embryo with hooks; thick, brown, radially striated embryophore
Hymenolepis nana	30–60 (diameter) Average 47 × 37	Nonoperculate; embryo with six hooks; poles of inner shell with filaments
Hymenolepis diminuta	70–85 × 60–80 Average 72 × 65	Nonoperculate; embryo with six hooks; poles of inner shell without filaments
Dipylidium caninum	20–50 (diameter) Average 40 × 36	Nonoperculate; embryo with six hooks; usually in packets of 10–12 ova

of the segments to break away and they may then be recovered; this procedure may be followed when the only proglottides passed are degenerate and not identifiable. The gravid proglottides and other sections of the adult worm may be identified by the key (Chapter 24). Thirteen or less main uterine branches on each side identify *T. solium,* fifteen or more *T. saginata;* the pinkish gravid proglottis with two gonopores and containing a large number of egg sacs is characteristic of *Dipylidium.*

Man's immunologic response to the intestinal tapeworms is generally slight, and serologic and allied tests have not proved satisfactory.

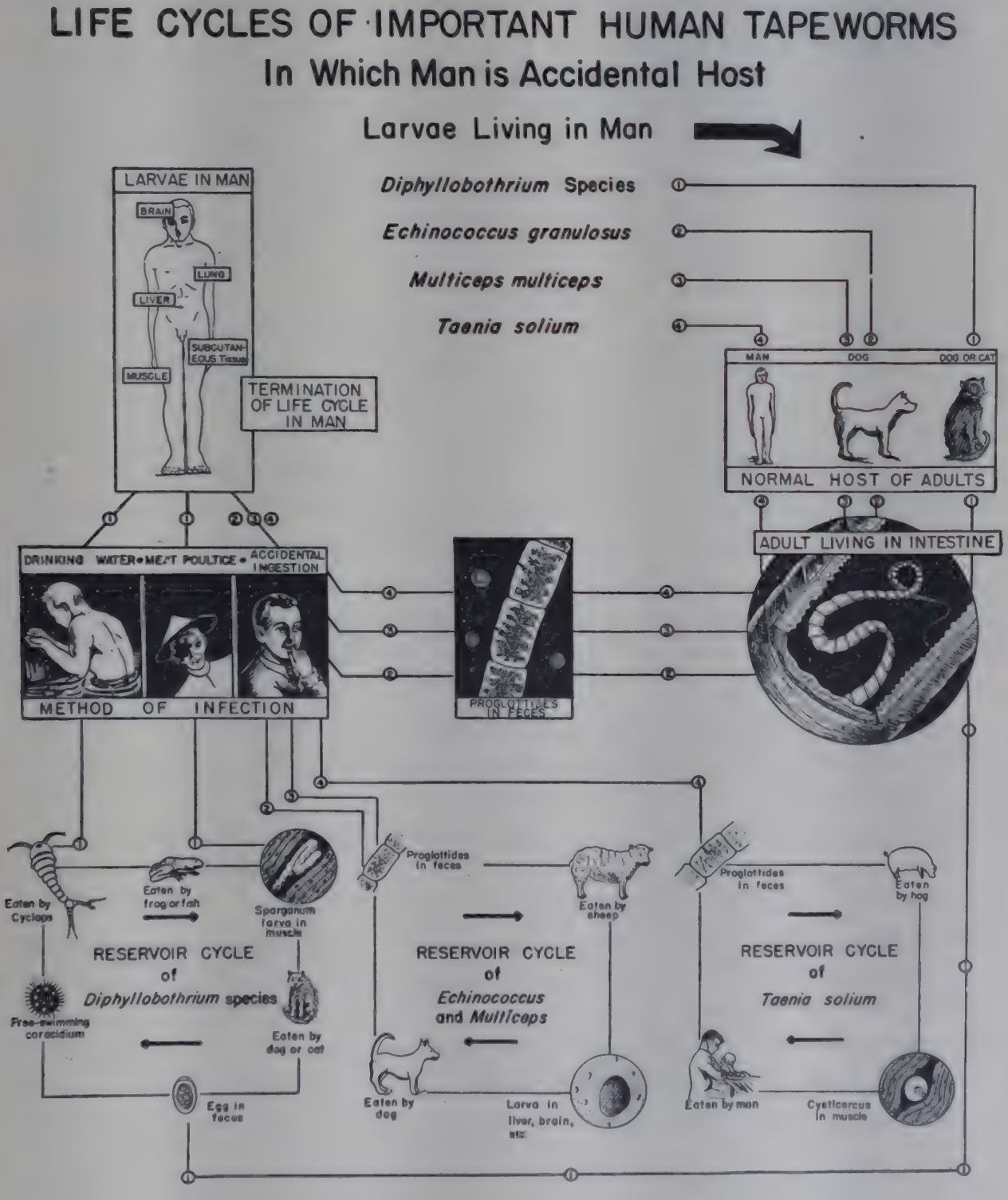
Table 65

DIFFERENTIAL CHARACTERS OF GRAVID PROGLOTTIDES

Species	*Size (in mm.)*	*Special Features*
Diphyllobothrium latum	2–4 × 10–12 "broader than long"	Rosette-shaped, coiled uterus; no lateral gonopore
Taenia saginata	16–20 × 5–7 "longer than broad"	15 or more main uterine branches; single lateral gonopore
Taenia solium	10–12 × 5–7 "longer than broad"	13 or less main lateral uterine branches; single lateral gonopore
Hymenolepis nana	0.15–0.3 × 0.8–0.9 "broader than long"	Uterus irregular, saclike; single lateral gonopore
Hymenolepis diminuta	0.75 × 2.5 "broader than long"	Uterus irregular, saclike; single lateral gonopore
Dipylidium caninum	10–12 × 2.5–3.0 "longer than broad"	Two gonopores on each segment; uterus with polygonal egg sacs

Somatic Tapeworms

Man serves as an accidental intermediate host for the larval stages of certain cestodes; the definitive host is a mammal, in one case man, which harbors the adult tapeworm in its intestine. The usual intermediate host must be slaughtered or die of disease, and its tissues containing the larval cestodes be devoured in order to complete the life cycle. Thus the parasite with the greatest pathogenicity and most often causing death has the best chance of transmission and survival. It



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Life cycles of important human tapeworms in which man is an accidental host.

is to be expected that highly virulent diseases should develop from this host-parasite relationship. The most severe diseases of man caused by cestodes fall into this group and it is fortunate that they are of rather limited occurrence.

Life Histories and Transmission. The invasion of the human body by cestode larvae has been observed in several species; their life histories are shown collectively. *Hymenolepis nana* might be included in this group but has been omitted because its larval stage is of little importance. Of the group to be discussed, there are several closely related species of the order Pseudophyllidea and three species of the order Cyclophyllidea. The life cycles of the former are very similar to that of *Diphyllobothrium latum*.

Eggs of the three cyclophyllidean tapeworms are discharged with the feces of their respective definitive hosts. When these ova are ingested by man, hatching occurs in the small intestine, the onchosphere penetrates the intestinal wall to reach the body tissues, and the larval stage develops. Here the life cycle ends unless human flesh is eaten by an appropriate definitive host. The customs of the human race have made this a very rare occurrence and, as a result, these larvae are usually in a blind alley or cul-de-sac in man.

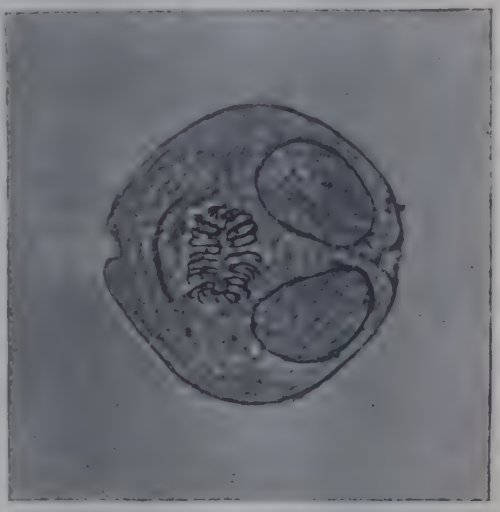
Prophylaxis. Infection by the somatic tapeworms is acquired almost exclusively by ingestion of the infective eggs; only in some cases of sparganosis is the parasite acquired by contact. Control depends primarily on good habits of personal cleanliness, and education of the population as to the dangers of too close an association with the reservoir host from which man is infected. Because of the great danger of self-infection, every case with *Taenia solium* should be treated immediately.

Artificial immunization of dogs and lambs against *Echinococcus* is a valuable control measure (Barnett, 1945). Treatment of dogs each year, slaughter of sheep at five months of age, and the migration of people from rural to urban areas have had great effect in the reduction of the incidence of echinococcosis in Iceland (Dungal, 1946).

Species: Echinococcus Granulosus. The usual hosts for the larval stages of this parasite are cattle, sheep, and hogs; the definitive hosts, the dog, wolf, or other carnivores. The infection occurs in man wherever he lives in such close association with the definitive hosts that his hands or food become contaminated with their feces. The disease *echinococcosis* is found in Iceland and Arabia and in parts of central Europe, North and South Africa, South America, New Zealand, and Australia. Dungal (1946) reports that at one time 50 per cent of all Icelanders were infected but that now the disease is seen only in the older people. It is rare in the United States although it has been reported to be common in hogs in some areas. The infection is also known as hydatid disease, from the type of encysted larva.

The adult worms are minute, 4 to 5 mm. long, and live in the small intestine of the definitive host. The head has four suckers and a rostellum encircled by two rows of hooks. There are usually three (sometimes four or five) segments, the terminal one larger than all the rest of the helminth and packed with eggs. The ova, found in the feces of dogs, closely resemble those of *Taenia saginata*. Upon ingestion the egg loses its membrane and the liberated embryo penetrates

the intestinal wall. It then usually passes to the liver (60 per cent), probably through the portal vein; it may be carried to lungs, kidneys, spleen, omentum, heart, brain, eye, or elsewhere but, as a rule, only one organ is involved. Within the organ penetrated the larva becomes enclosed in a cyst wall made up of two layers; the outer layer is thick, laminated, and elastic so that it curls up when incised; the inner layer is made up of a protoplasmic matrix containing numerous nuclei. A protective capsule is formed around the cyst by the tissues of the host. Bud-like processes arise from the inner or germinal layer; these become vesicular and are known as *brood capsules*. Numerous *scolices* are produced by a process of localized proliferation and invagination of the wall of the brood capsule. Each is about 0.175 mm. in diameter, is borne on a pedicle, and shows suckers and two rows of hooklets. Some of the brood capsules separate from the wall and settle to the bottom of the cyst as a fine, granular sediment known as "hydatid sand"; liberated scolices may also be present.

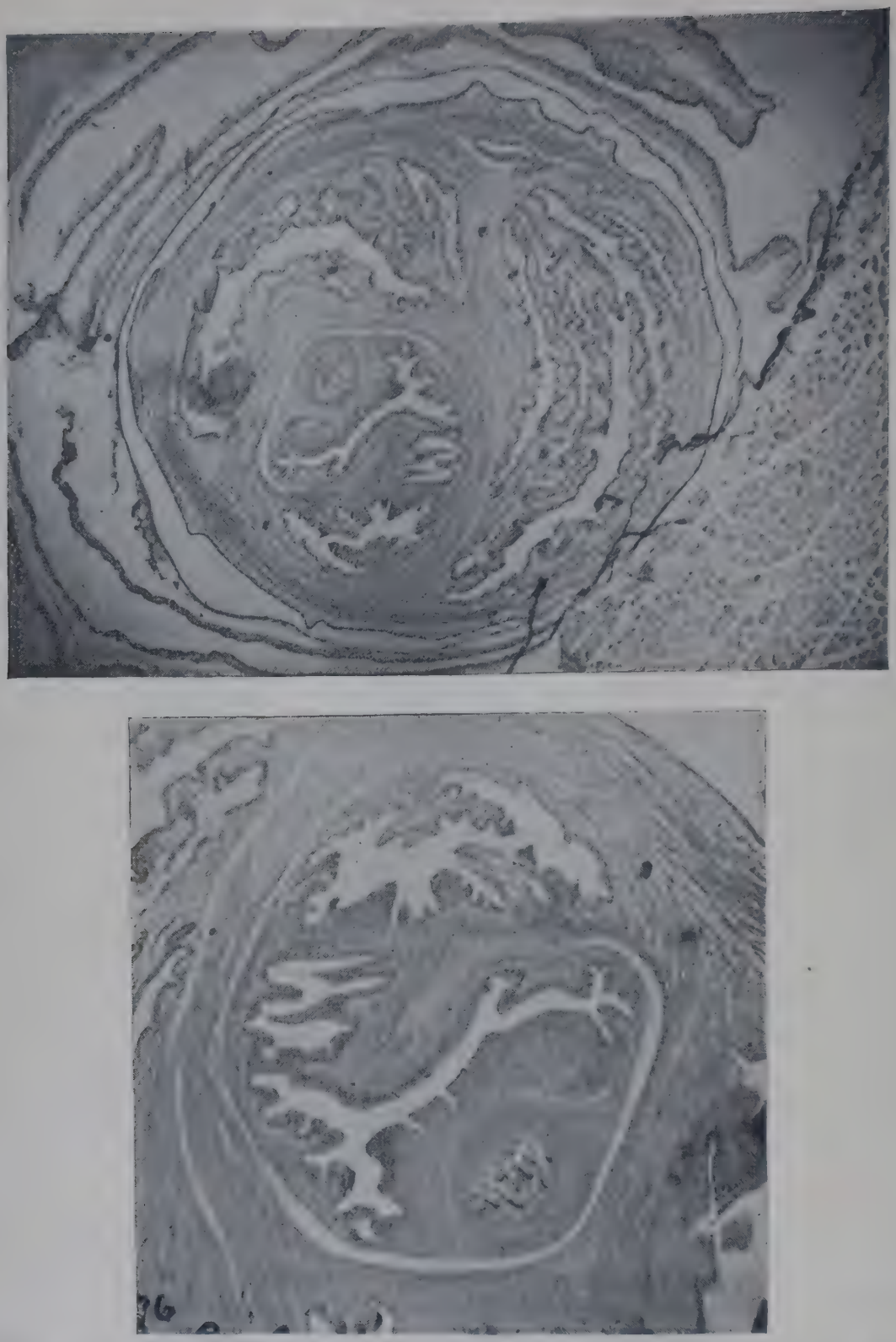


Single scolex of *Echinococcus granulosus*. (Photomicrograph × 200.)

As the hydatid cyst gradually enlarges, reaching a diameter of about 10 cm. after 10 months, invagination of the wall may give rise to daughter cysts (possessing a laminated external layer as well as a germinal layer) but such secondary cysts usually arise by development from brood capsules or scolices. Granddaughter cysts may arise from them in a similar manner. There is a continuous formation of brood capsules and scolices within each cyst. Growth may continue for several years, and the cyst may attain the size of a child's head; usually it is smaller. It has been estimated that as many as 2,000,000 scolices may thus develop from a single ovum. Occasionally a cyst is barren, containing no scolices. When the contents of the cyst are eaten by the definitive host, each scolex may develop into an adult worm.

In some cases no effective encapsulation occurs, and the daughter cysts develop by evagination of the cyst wall. This results in the formation of a mass of small, discrete vesicles, much like a bunch of minute grapes, which tend to infiltrate the surrounding tissues and even to metastasize to other organs like a malignant tumor ("Gallertkrebsen"). These occur most often in the liver, especially in cattle. Because such *multilocular* or *alveolar* hydatids are common in man in certain districts (southern Germany, Russia) but are almost unknown in others, the parasite has been regarded as a distinct species, *E. multilocularis;* present evidence is against this concept. When the larva becomes localized in bone, a third type, the *osseous cyst,* may develop.

Taenia Solium. The larva of this cestode is known as *Cysticercus cellulosae* and produces the disease known as *cysticercosis*. Eggs may be ingested by man, or regurgitated into the stomach from an adult worm situated in the intestine; they



Cysticercus larva of *Taenia solium*. (*Top*) Entire larva. (*Bottom*) Section of larva to show hooks. (Photomicrographs (*top*) × 10, (*bottom*) × 20.)

then undergo a development with an invasion of tissue similar to that which occurs in hogs. Any organ may be involved, frequently the brain and occasionally the eye.

Investigation of cysticercosis in Mexico and in English soldiers returning from India has stressed autoinfection or possibly hyperinfection as the source of the disease. Dixon and Hargreaves (1944) in a study of 284 cases found that 26 per cent had a history of infection by the adult *T. solium*. It then becomes very

important to recognize the proglottides of *T. solium* when present in the feces, to enforce rigorous precautions to avoid ingestion of eggs, and to institute prompt treatment.

Diphyllobothrium Species. The plerocercoid or sparganum larvae of certain species of *Diphyllobothrium* cause a disease in man known as *sparganosis*. *D. mansonoides* and related species are pseudophyllidean tapeworms which are found as adults in dogs, cats, and other carnivores; sparganosis occurs most commonly in the Orient but is found in other regions of the world, including Africa, Holland, and the United States. Man acquires the larval stage in one of several ways: (1) by drinking water containing microcrustaceans infected with the procercoid larvae; (2) by the use of fresh frog tissue as a poultice for sore eyes and wounds; the plerocercoid or sparganum larvae then are transferred to human flesh; (3) by accidentally ingesting the plerocercoids when handling infected frogs or fish.

Clinical Illness: Echinococcosis. Echinococcosis of the liver is usually symptomless in the early stages; a tumor may be palpable. Large cysts on the convex surface may be mistaken for a pleural effusion. The symptoms are those of a liver abscess when the contents become infected. Rupture may occur spontaneously into the peritoneal cavity, pleural cavity, or lung; less frequently into the gastrointestinal tract or other tissues. Cysts which do not rupture eventually cause grave injury as a result of pressure and destruction of tissues. Anemia, emaciation, and weakness develop, and death usually occurs within a few years unless complete surgical removal is accomplished. Barnett (1945) emphasizes the danger of tapping an echinococcus cyst and gives an excellent summary of the knowledge of the disease. He gives the dictum, "a hydatid cyst is nearly as old as its host."

Involvement of the lung occurs in about 12 per cent of the cases and is more serious than cysts of the liver. Early symptoms include cough, small hemoptyses, bouts of fever, and transient physical signs, all suggesting early tuberculosis. Later symptoms suggest a tumor or abscess. Rupture may occur into a bronchus or into the pleural cavity. If such an accident is not quickly fatal, recovery may occur spontaneously or after surgical drainage. Eosinophilia is usually present. Barrett and Thomas (1944) believe the lung cysts should never be aspirated. Occasionally cases of hydatid of the lung are seen in the United States (Mathiesen, 1947).

Hydatid of the bone always leads to a grave prognosis; in the spine, Castro (1944) believes it to be uniformly fatal.

Cysticercosis. Cysticercosis will vary in severity, depending upon the tissue or organ parasitized and the number of larvae present. In a favorable site the larvae will eventually die and become calcified with few or no symptoms. A single cysticercus in the brain or eye may obviously produce severe damage; those in the brain may cause a Jacksonian type of epilepsy.

Sparganosis. Sparganosis may produce local pathologic lesions at the site of infection. Involvement of the ocular region may be serious. Lesions of the skin are less important unless the number of larvae is large.

Diagnosis. Serologic and allied tests offer the most practical method of diagnosis. Since the somatic forms cause profound tissue damage and produce a powerful

immune response, such tests are much more important in these diseases than in the intestinal infections. The reactions are of a group character and must be carefully evaluated. In some cases a negative reaction does not exclude the possibility of infection.

The most delicate and specific test for echinococcosis is the intradermal or Casoni reaction. Complement-fixation and precipitation tests are also available but are of less value except in old or complicated cases. Similar methods are used with somewhat less reliable results in the diagnosis of cysticercosis. Sparganosis has been successfully diagnosed by serologic tests in monkeys and in a few instances in human beings (Mueller and Coulson, 1942). Grana (1945) gives an excellent summary of the serologic methods of diagnosing echinococcosis.

In cases where a lung cyst has suppurated or a liver cyst has ruptured into a lung, fragments and hooklets may be found in the sputum. Exploratory aspiration is dangerous; if leakage of fluid occurs or if the cyst ruptures, a violent reaction follows, anaphylactoid in type. The scattered scolices tend to become implanted and give rise to new cysts. Again the diagnosis is made from the identification of free scolices or of scattered hooklets from those that have disintegrated.

The cysticercus larvae may be diagnosed after biopsy or at autopsy by the typical miniature scolex of *Taenia solium*. Very often it is impossible to excise the encysted larvae because of their location in the body. Roentgen-ray examination is of value, especially where calcification has occurred. Hydatid cysts of the lung are characterized by their sharp outline.

CHAPTER 23

The Roundworms or Nematodes

The nematodes are among the most ubiquitous of all invertebrates, enormous numbers of free-living forms inhabiting almost every possible environment in addition to the large number of parasitic species. They have a simple organization which combines high resistance to unfavorable environment with great powers of reproduction. As a consequence they are one of the more successful groups of animals but are remarkable in that they show little similarity to any other animal phylum. The roundworms are the most common and important helminth parasites of man.

Classification. The classification of the Nemathelminthes has always been difficult because it involves the correlation of the free-living roundworms, many of which are inadequately known, with the parasitic forms. The system most generally followed is that of Chitwood (1936) which divides the nematodes into two groups, mainly on the basis of their sense organs and excretory systems. The first group, *Aphasmidia,* includes forms that are primarily aquatic, among which few parasites of man have evolved. The second group, *Phasmidia,* contains species that are fundamentally soil-inhabiting and also the great majority of roundworms parasitic in man. Only members of the order Enoplida belong to the first group.

It is more practical for the student or technician to consider the nematodes under two groups: *intestinal nematodes,* including forms in which the adult worm lives in the intestine or intestinal mucosa, and *somatic* or *tissue nematodes,* including species in which the adult inhabits the tissues of man, other than the intestinal mucosa. *Trichinella spiralis* is more or less unique; although the adults live in the intestinal mucosa, it differs from the other species in life history, clinical illness, prevention, and diagnosis and will be considered separately.

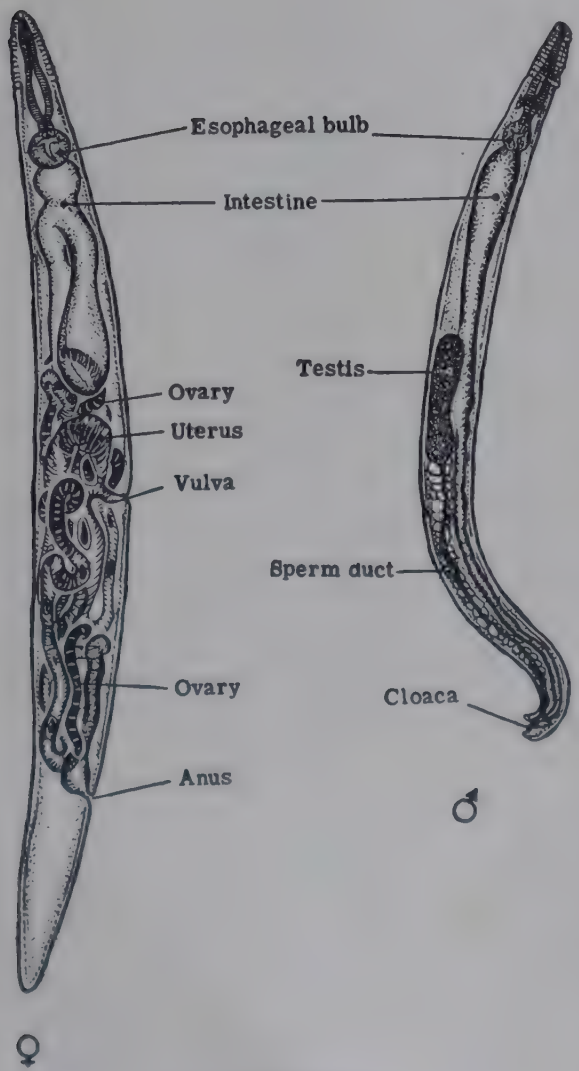
Intestinal Nematodes:

Trichuris trichiura (Linnaeus, 1771) Stiles, 1901
Strongyloides stercoralis (Bavay, 1876) Stiles and Hassall, 1902
Ancylostoma duodenale (Dubini, 1843) Creplin, 1845
Ancylostoma caninum (Ercolani, 1859) Hall, 1913
Ancylostoma braziliense de Faria, 1910
Necator americanus (Stiles, 1902) Stiles, 1903
Ternidens deminutus (Railliet and Henry, 1905) Railliet and Henry, 1909
Trichostrongylus colubriformis (Giles, 1892) Ransom, 1911
Trichostrongylus orientalis Jimbo, 1914
Enterobius vermicularis (Linnaeus, 1758) Leach, 1853

Ascaris lumbricoides Linnaeus, 1758
Gnathostoma spinigerum Owen, 1836
Physaloptera caucasica v. Linstow, 1902 (only immature form in man)

Intestinal and Somatic Nematodes:
Trichinella spiralis (Owen, 1853) Railliet, 1895

Somatic or Tissue Nematodes:
Wuchereria bancrofti (Cobbold, 1877) Seurat, 1921
Wuchereria malayi (Brug, 1927) Rao and Maplestone, 1940
Onchocerca volvulus (Leuckart, 1893) Railliet and Henry, 1910
Loa loa (Cobbold, 1864) Castellani and Chalmers, 1813
Acanthocheilonema perstans (Manson, 1891) Railliet, Henry and Langeron, 1912
Mansonella ozzardi (Manson, 1897) Faust, 1929
Dracunculus medinensis (Linnaeus, 1758) Gallandant, 1773



A nematode.

In addition to the above twenty-one species which have been reported from man in many instances, there are approximately forty-five other nematodes which are rare, accidental, or spurious parasites of man.

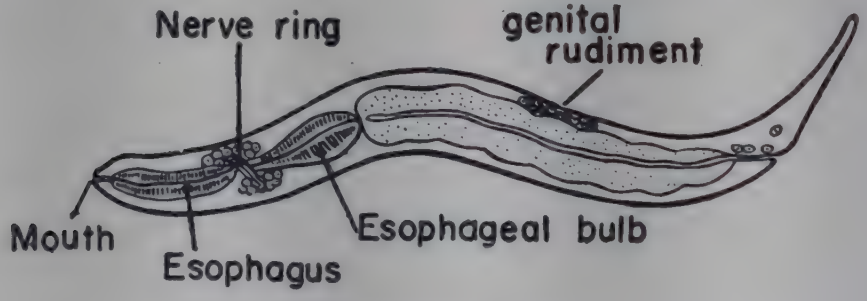
Morphology. The adult nematodes of man may be easily distinguished from all other helminths by the following characters: (1) body threadlike and cylindrical, round in cross section; (2) neither an internal nor an external segmentation; (3) separate sexes (dioecious); (4) complete digestive tract with mouth and anus. The entire body is covered by a hyaline, noncellular, homogeneous and highly impervious layer, the *cuticle*. The underlying *hypodermis* which secretes the cuticle is usually markedly thickened internally to form four longitudinal ridges which divide the body into quadrants. A single layer of *longitudinal muscles* forms the innermost layer of the body wall; by alternate contraction and relaxation these muscles give the nematode its very characteristic, snakelike movement. Nematodes which live in the tissues of man require no attachment organs, but those which inhabit the lumen of the bowel maintain their position in one of three

ways: by their own movements (*Ascaris, Enterobius*), by mouth structures (*Necator, Ancylostoma*), or by actual penetration of the intestinal mucosa; the entire worm may enter the intestinal wall (*Trichinella, Strongyloides*) or only the anterior portion of the body (*Trichuris*).

The nervous and excretory systems are very poorly developed. The former consists of longitudinal nerves united near the anterior end by a *ring commissure.* Special sense organs, chemo- and touch-receptors, are located at the anterior and posterior ends of the body. The *excretory system* usually consists of two lateral, longitudinal tubules opening to the exterior by a ventral pore just posterior to the esophagus; it may be greatly reduced in some species.

While the alimentary canal is tubelike in appearance, it shows a distinct *esophagus* near the mouth. The *buccal capsule* and esophagus are lined by a continuation of the cuticle which forms teeth and other structures in some species.

The sexes are separate, almost without exception, and the *testes* and *ovaries* are generally tubelike. The male can usually be recognized by its smaller size and its curved or curled posterior end, at times exhibiting an umbrella-like expansion—the *copulatory bursa.* The genital opening of the female is ventral and may vary in position from close to the mouth to near the tail. The genital pore of the male is adjacent to the anus, both opening into a common *cloaca* with a cloacal opening in the ventral line; the *spicules*—chitinous copulatory structures—may be



Rhabditiform larva.

observed drawn up in the body or projecting out of the cloaca. Most nematodes produce eggs which hatch after being laid (oviparous), but in some the eggs hatch within the uterus (ovoviviparous). The state of development of ova which are laid varies greatly; some (*Ascaris, Trichuris*) are unsegmented, others (hookworms) are in an early stage of development, and one (*Enterobius*) has almost reached the infective larval stage.

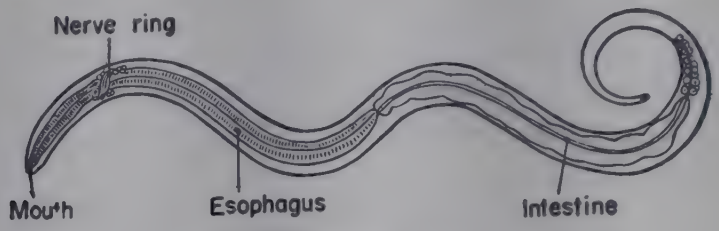
The cuticle of a roundworm is molted or shed at intervals throughout its life; there are typically four molts. The worm is known as a larva until after the fourth molt when it is an adult. Larvae differ from the adults primarily in size and in development of reproductive organs. The immature stages of some species (hookworms, *Strongyloides,* filariae) are important in differentiation of species and more definitive names have been applied to them.

Rhabditiform Larva. This term is used to designate the first larval stage of the hookworms, *Strongyloides,* and others. The name refers to the structure of the esophagus which is composed of two portions, an anterior tubular part

separated from a posterior bulb by a constriction or neck. This is typically a non-infective form which feeds on bacteria and organic material.

Filariform Larva. This term is used primarily for designation of the infective larval stage of the hookworms and *Strongyloides*. In the filariform larva the esophagus is long and cylindrical, without the terminal bulb. This larva is unable to feed.

Microfilaria. This is the embryo discharged from the uterus of the adult female filarial worm. It may or may not have a sheath; some authorities believe the sheath to be the elastic egg shell, others that it is a cuticle retained after molting as is seen in the filariform larvae of the hookworms.



Filariform larva.

Intestinal Roundworms

Many of the intestinal nematodes may be encountered wherever man exists. The peculiarities of certain species may modify the incidence, an unfavorable environment for the free-living stages being an important factor.

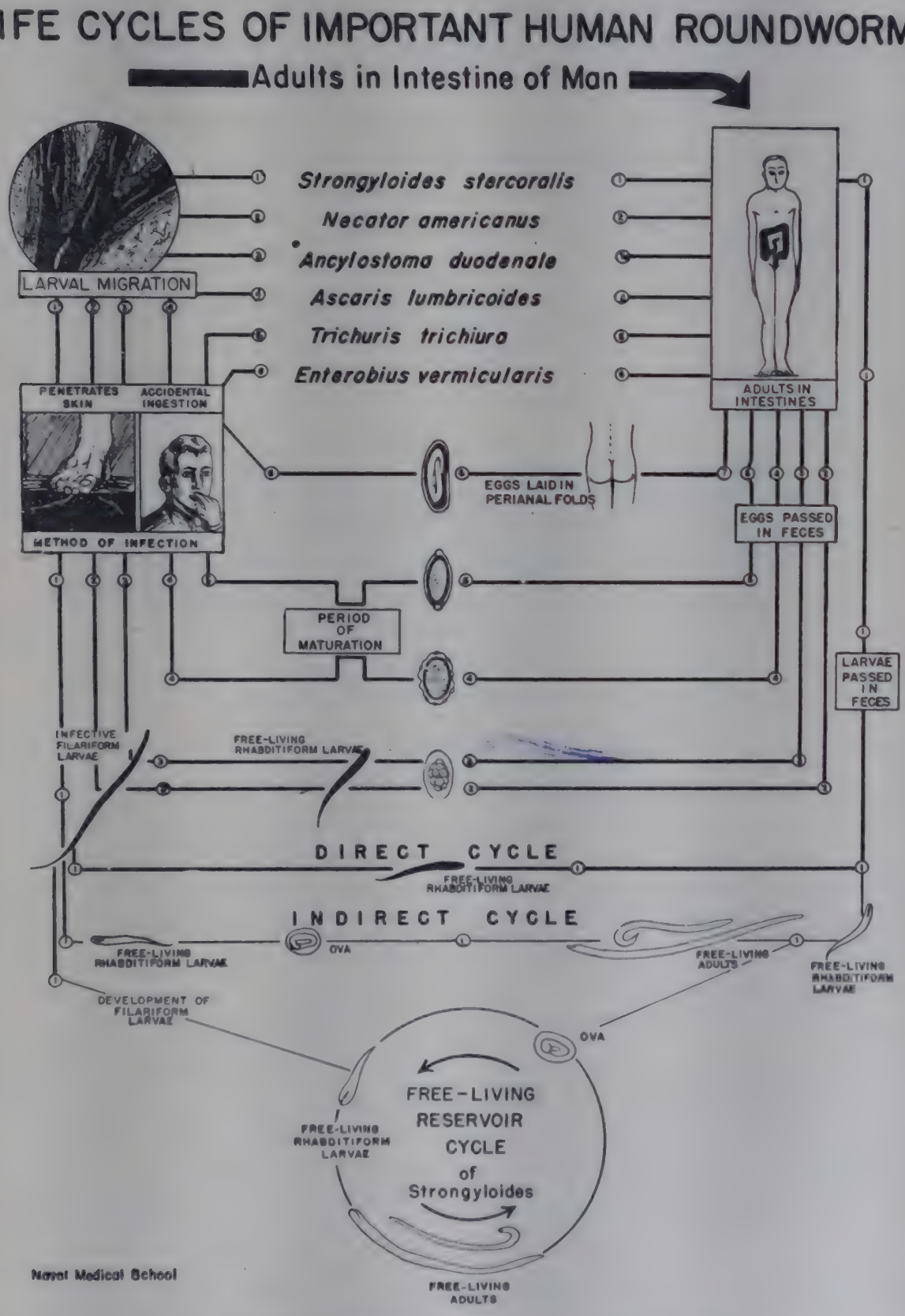
Life Histories and Transmission. When the life histories of the more common of these helminths are depicted together it is found that they form a series which illustrates some of the possibilities which may occur in the evolution of parasitism. In the order of increasing adaptation, the series passes from *Strongyloides* through *Necator, Ancylostoma, Ascaris,* and *Trichuris* to *Enterobius*. Inasmuch as none of these species requires an intermediate host the only differences in their life cycles are the stages and periods of time spent outside man and their larval migrations within man. The most primitive form, *Strongyloides,* is able to maintain itself in a free-living cycle; the parasitic cycle is initiated when the rhabditiform larva becomes filariform and penetrates the skin of man with subsequent migration. The next forms, *Necator* and *Ancylostoma,* have no free-living adults although their eggs and two larval forms live outside the host; again the filariform larva penetrates the skin and migrates as in *Strongyloides*. *Ascaris* forms the third step in the series; only the eggs live outside man, requiring about three weeks in warm, moist soil before they become mature and infective. The embryonated egg must be ingested and hatches in the intestine; this is followed by a larval migration similar to that of the above species.

The fourth step is *Trichuris* which is similar to *Ascaris* in requiring an incubation period for the egg outside the host; it is different in that the larva which escapes from the egg after ingestion attaches itself to the mucosa without migration. The last example is *Enterobius* in which the eggs are practically mature and infective when laid on the perianal skin. There is no larval migration, adults developing from the larvae which are released from ingested eggs.

While the above roundworms have only a definitive host, a few intestinal

nematodes (Spiruroidea) also have an intermediate host, usually an insect in which the larva develops. The cycle is completed when the insect containing the infective larva is ingested, accidentally in the case of man.

Prophylaxis. All of the intestinal nematodes, with the exception of *Enterobius vermicularis,* have life cycles which can be interrupted or controlled by the obvious, practical measure of sanitary disposal of feces. Treatment of infected persons

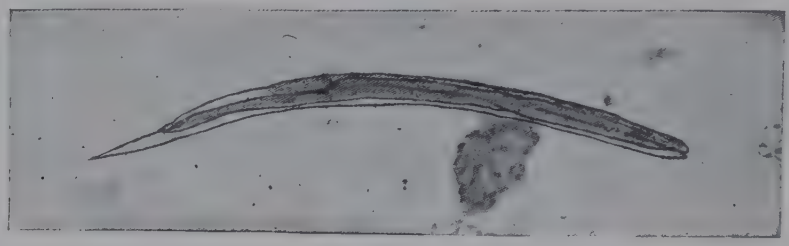


Life cycles of important human intestinal roundworms.

can be an important adjunct. Except for the hookworms and *Strongyloides,* all enter man via the mouth. Careful protection of food and drink from contamination will suffice in these instances, even though adequate disposal of human excreta has not been established. The name "geohelminth" has been applied to these species which are transmitted by soiled articles. The contaminated person and clothing of the carrier have been shown to be of primary importance in their transfer (Golubiatnikova, 1943). Infection by hookworms and *Strongyloides* is prevented by avoiding contact with contaminated soil—often moist, shady, sandy areas. In many cases this end may be attained by simply wearing shoes.

The ova of the pinworm are usually not present in the feces. Personal cleanliness is the important prophylactic measure for this species; the eggs are known to be widely scattered in the environment of the infected person.

Species: Strongyloides Stercoralis. This parasite is especially prevalent in Cochin-China and Brazil, is widely distributed in tropic and semitropic regions, and is fairly common in the southern United States. It is dependent upon a warm, moist soil for the completion of its life cycle. Its distribution is similar to but not so extensive as that of the hookworms parasitic in man; the limitation is probably due to the rather poor resistance of the larvae to desiccation, excessive humidity, and marked changes in temperature.



Rhabditiform larva of *Strongyloides stercoralis.* (Photomicrograph × 200.)

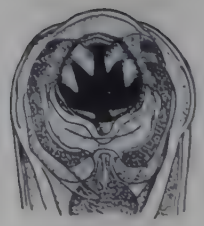
There are two types of females, a parasitic intestinal and a free-living form, which differ strikingly in morphology. (See table below.)

The parasitic and free-living males are very similar, with an esophageal bulb and a curved, pointed tail. The similarity of the two male forms together with the elucidation of the life cycles of closely related species, such as *S. ratti* which has only parthenogenetic females, has led some authorities to doubt the function of the parasitic males. The most widely accepted life history is described below.

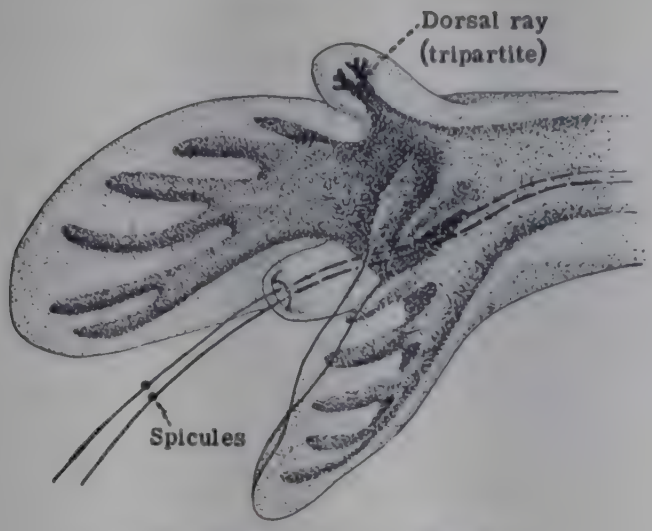
Distinguishing Features of Females of Strongyloides Stercoralis

	Parasitic form	*Free-living form*
Length	2–2.5 mm.	About 1 mm.
Width	40–50μ	50–60μ
Esophagus	One-fourth body length; no terminal bulb (filariform)	One-fifth body length, with terminal bulb rhabditiform)
Vulva	Near posterior third of body	About middle of body

ANCYLOSTOMA DUODENALE

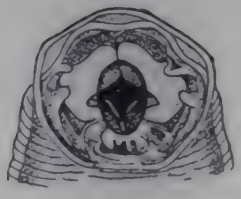


BUCCAL CAPSULE

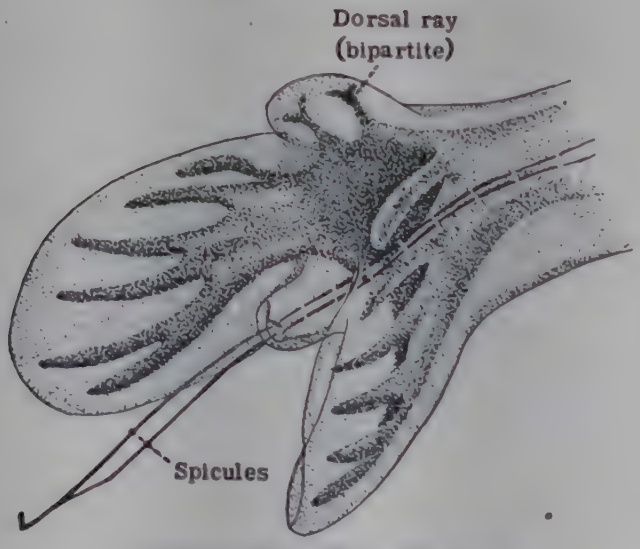


COPULATORY BURSA OF MALE

NECATOR AMERICANUS



BUCCAL CAPSULE



COPULATORY BURSA OF MALE

NAVAL MEDICAL SCHOOL '44

Differential characters of important human hookworms.

The parasitic females lay ova which quickly develop and the rhabditiform larvae are hatched in the intestine. In very rare instances in severe diarrhea or after brisk purgation, eggs have been recovered from the stools. When first hatched the larvae measure about 0.25 mm. in length, but by the time they are passed in the feces they may have grown to twice that size.

In direct transmission back to man the rhabditiform larva metamorphoses into an infective filariform stage after a brief period of growth; the latter is ensheathed by the cuticle of the previous larval stage. Under certain conditions, possibly constipation or regurgitation, rhabditiform larvae develop directly into the filariform type within the body of the host; these may then penetrate the intestinal mucosa at the same level, at a lower level, or in the perianal region and initiate a new infection (hyperinfection). Indirect transmission may follow when the rhabdit

iform larvae develop into free-living males and females; infective forms may then develop at any time from the rhabditiform larvae produced by this non-parasitic generation.

The usual infection of man takes place when the filariform larvae penetrate the skin or when they penetrate the mucosa after ingestion. They then pass through the blood stream to the lungs where they remain for some days, developing into adolescent males and females. Some worms start reproduction while in this site. Most of them then pass by way of the bronchi and trachea into the esophagus and down to the intestine where the female bores into the mucosa and begins to deposit ova, about two weeks after infection.

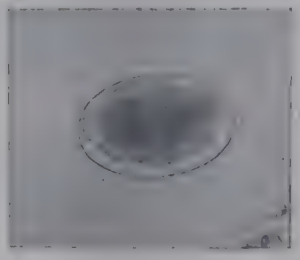
Hookworms—Ancylostoma Species and Necator Americanus. Intestinal hookworm infections of man are nearly all caused by two species: *Ancylostoma duodenale,* the "Old World" species, and *Necator americanus,* the "New World" species. Actually both are widely distributed throughout tropic and subtropic regions and are found in the temperate zones in mines, tunnels, and other places in which warmth and moisture are available. *A. duodenale* is found principally in southern Europe, northern Africa, China, and Japan; *N. americanus* occurs in the southern United States, Central America, and the Caribbean Islands, northern South America, central and south Africa, southern Asia, and Polynesia.

The adult worms are found in the small intestine (jejunum) of man, sometimes in enormous numbers—1500 or more. They attach themselves to the mucosa from which they suck blood. The more common morphologic features are listed below.

Distinguishing Features of the Common Hookworms

	Ancylostoma duodenale	*Necator americanus*
Position of head	Continues in same curvature as that of body	Turned dorsally and at an angle with general curvature of body, giving hooked appearance to anterior end
Buccal capsule	Two pairs of ventral, clawlike teeth, one pair of dorsal knoblike teeth	Two ventral cutting plates and two rudimentary dorsal plates
Male		
Size	8–11 mm.	5–9 mm.
Dorsal ray of copulatory bursa	Divides in the distal third, each division ending in three digitations (tripartite)	Divides at the base, each division ending in two digitations (bipartite)
Spicules	Two; separate, hairlike	Two; hairlike, fused and barbed at tip
Female		
Size	10–13 mm.	9–11 mm.
Posterior tip	With spine	Without spine

The life history is practically the same in both species of hookworms. The eggs laid by the adult females do not undergo segmentation within the intestine, probably because of lack of oxygen. After being passed, development progresses rapidly in the presence of warmth (14° to 37° C., preferably 27° C.), moisture (muddy water, damp earth), and oxygen so that small *rhabditiform larvae* are hatched within two days. These feed voraciously, grow rapidly, and reach a length of 0.3 mm. on the third day when they molt; on the fifth day the larvae have reached a length of 0.55 mm. and they molt again, losing the bulblike swelling of the esophagus and becoming *filariform larvae*—the infective stage. The old cuticle is not shed but is retained as a protecting sheath. The parasites then enter into a resting stage in which they cease to take food although they remain actively motile and can crawl up blades of grass or the vertical sides of mines. The larvae usually do not migrate more than a few inches from the place of development. Before entering the resting stage, they are readily destroyed by direct sunlight, by chemicals, or even by dilution of feces, especially with urine. After molting they become relatively resistant and may live four to eight weeks in shaded, moist ground in the tropics; in a cool, moist environment they may live much longer but are quite sensitive to desiccation and extremes of temperature.



Ovum of hookworm. (Photomicrograph × 200.)

When infected soil comes into contact with the body of the host the larvae become very active, probably stimulated by the warmth, and burrow through the skin. Apparently any part of the skin can be penetrated, and only a few minutes are required for the process. In the subcutaneous tissue the larvae enter the veins and lymphatics and are carried passively to the lungs; here they penetrate the walls of the capillaries and alveoli to enter the air sacs and proceed or are carried up the bronchi and trachea to the pharynx. They are then swallowed by the host and reach the intestine about one week after entering the skin, having undergone a third molt during migration. Maturity is reached in another three weeks, the fourth molt occurring in the intestine (when the parasite is about 2 mm. long). The exact duration of life is not known but probably varies from a few months to six years. Infection can result if larvae are swallowed. Fulleborn has shown that a few larvae can reach the intestine without passing through the lungs in experimental animals.

Ancylostoma Braziliense. *Ancylostoma braziliense,* and, less commonly, other hookworms (*A. caninum, Uncinaria stenocephala,* and others) which are normally parasitic in dogs and cats may produce infections of the skin of man. The disease known as *creeping eruption,* sometimes called larva migrans, is caused by the infective filariform larvae of these species; they seem unable to develop in man, an abnormal host, and wander about beneath the surface of the skin, eventually dying. However, *A. braziliense* can develop and exist as an adult in the human intestine in some areas of the world; apparently there are different strains of this parasite, differing in their adaptability to various hosts. Man is infected when he is exposed to the mature filariform larvae in moist, shady soil where dogs or cats have defecated.

Ternidens Deminutus. This is a small roundworm about the size of a hookworm. Its terminal buccal capsule is surrounded by a crown of leaflets; at the base there are three forked teeth guarding the entrance to the esophagus. The vaginal orifice is near the posterior tip. The ova resemble those of the hookworms but are larger, more segmented, and somewhat flattened on one side with broadly rounded poles. This parasite occurs in several monkeys and has been found not uncommonly in man in south Africa (Sandground, 1931; 1932). The adult worms inhabit the large bowel, rarely producing symptoms. The life cycle is not completely known, but infection does not seem to occur by way of the skin.

Species of Genus Trichostrongylus. *Trichostrongylus orientalis, T. colubriformis,* and related species of this genus are usually parasitic in the intestines of cattle, sheep, and other ruminants. They are not uncommon in man in certain areas of the world, especially the Orient, India, and the Near East. Infection by these helminths has been reported from the United States, and new reports may be expected with the large number of troops returning from the Orient. *Trichostrongylus* ova have been found in stool specimens of two patients at the Naval Medical School recently. Reports of *Trichostrongylus* species from man are summarized by Watson (1946).

The adults are long, slender worms with very poorly developed buccal capsules. The females measure about 5 to 7 mm. in length and the males 4 to 6 mm. The latter have fan-shaped copulatory bursae similar to those of the male hookworms; separation of the species can be most easily made on characters of the male bursa and spicules. The ova are larger than those of the hookworms and usually contain morula-stage embryos when passed; they are highly translucent and have one pole more pointed than the other. The eggs of the different species are practically indistinguishable.

Infection is caused by ingesting larvae, rarely by penetration of the skin. There is no migration within the body of the host. The first larval form, hatched from the egg, resembles the rhabditiform type and has a long buccal cavity; the tail terminates in a minute knob. The infective larva, resembling the filariform type, has a tuberculate tail. These parasites usually cause no symptoms, although it is reported that heavy infections may cause severe secondary anemia.

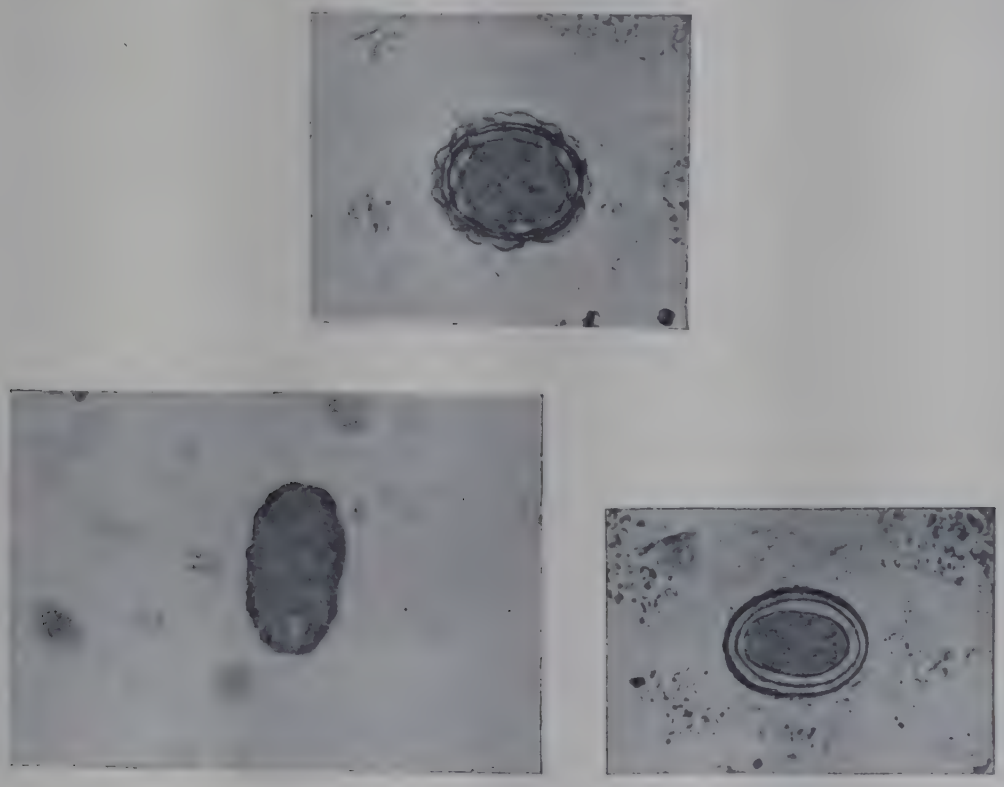
Ascaris Lumbricoides. The common roundworm or eelworm is one of the most prevalent parasites of man, especially of children. It occurs throughout the world, in temperate as well as tropic regions, and has been reported from the Arctic Zone. The *Ascaris* of the pig, morphologically indistinguishable, is apparently not adapted for parasitic life in man.

The adult worm is cylindrical, tapering to a blunt point at each end; the body is transversely striated and yellowish-gray to light brown in color. The female is usually 20 to 35 cm. long (occasionally up to 45 cm.) and 4 to 5 mm. in diameter; the male is more slender and from 15 to 30 cm. long. The mouth is provided with three papillae-like lips with finely denticulate margins and marked off from the rest of the body by a sharp constriction. The male has a cloaca near the posterior tip; from it project two large lancelike copulatory spicules. The posterior extremity is ventrally curved in the male and straight in the female. The uterus consists of long, whitish, convoluted, threadlike tubes which terminate in oviducts leading to the vulval orifice on the ventral surface at the junction of the anterior and middle thirds of the body.

The female discharges enormous numbers of ova after fecundation, estimated at 200,000 daily. These are elliptical and provided with a thick, smooth, trans-

lucent inner membrane and a rough, mammillated outer coat. The contents are granular, not segmented, and usually show a clear crescentic area at each pole. Eggs which are deposited *unfertilized* are markedly abnormal in their appearance, often longer, irregular and sometimes grotesquely misshapen, with structureless granular contents so that they may be mistaken for vegetable cells. At times the outer mammillated coat of either the fertile or infertile ovum is lost. These eggs, usually spoken of as *decorticated,* are distinguished by their thick, clear shell and internal structure.

The adult worm lives in the upper small intestine, and unsegmented, noninfective eggs are passed in the feces. In the presence of warmth (but not at body



Ova of *Ascaris lumbricoides.* (*Top*) Fertile ovum. (*Lower left*) Infertile ovum. (*Lower right*) Decorticated ovum. (Photomicrographs × 200.)

temperature), a little moisture and oxygen segmentation occurs; development of the embryo is completed outside the body after an interval of from 20 to 40 days or more (rarely less than 30), depending upon the temperature—optimum 25° C. The larva molts once within the shell and normally remains there until ingested; however, hatching can be induced by first desiccating and then moistening the ovum, and the larva may live for several weeks in moist earth. The ova are very resistant to the usual chemical disinfectants, to cold (down to —15° C.), and to desiccation at moderate temperatures but are killed by drying in hot weather. Eggs have remained viable for four years at icebox temperature when kept moist, two years when dried. Infection results from swallowing embryonated ova.

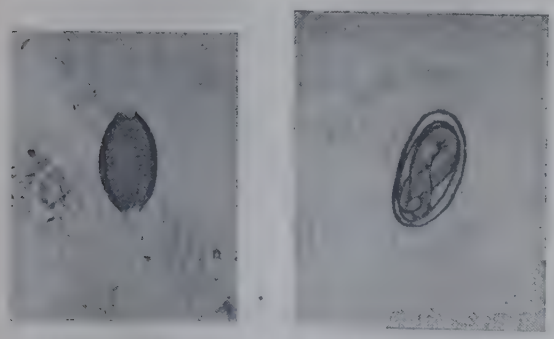
The eggs may be conveyed to the mouth by dirty fingers (especially in children) or by contaminated water, green vegetables, or other food, and possibly by inhalation. In regions in which soil pollution is common or in which human feces are

used as fertilizer, infections may be heavy. The larvae, 0.25 mm. long, are liberated in the lumen of the small intestine. They penetrate the wall of the gut and pass to the liver, usually through the portal circulation, perhaps a few by direct migration through the peritoneal cavity. After three to four days they reach the lungs and penetrate the walls of capillaries and alveoli. After a further period of development of about 10 days and involving two molts, they enter the air passages, pass by way of the trachea to the pharynx, and are swallowed as in the case of the hookworms. Passage through the lungs seems to be essential for the development of the adult. On reaching the intestine, the larvae have attained a length of about 2 mm. and develop into adult forms in a period of 8 to 10 weeks. The life span has been estimated as about one year.

Trichuris Trichiura. The whipworm is one of the most common parasites of man in both temperate and tropic climates. Man is the only proved host of *Trichuris trichiura* although whipworms obtained from the pig and certain monkeys are morphologically very similar and are believed by some to be the same species. The generic name *Trichocephalus* is an equally valid one, preferred to *Trichuris* by some authors. While the International Commission on Zoological Nomenclature has not ruled on the two names, the American Society of Parasitologists has expressed a preference for *Trichuris*.

The adult worm is 30 to 50 mm. long, the female being slightly longer than the male. The cephalic half to two-thirds of the body is threadlike and contains only the esophagus; this has reduced musculature which is apposed to a row of large secretory cells, the stichocyte, communicating with the lumen of the esophagus by small apertures. A stylet is present at the anterior end. The caudal portion of the body—the handle of the whip—contains the intestine and sex organs. The tail of the male is coiled and ends in a single terminal spicule surrounded by a rough, spiny sheath. The tail of the female is bluntly rounded; the vulva opens at the junction of the "handle" and the "lash."

Whipworms are found chiefly in the cecum, but also in the appendix and terminal ileum, where they attach themselves to the intestinal wall by transfixing a fold of mucosa with their slender neck; this has been thought by some to facilitate the entrance of typhoid bacilli and other pathogenic bacteria into the tissues. The characteristic, unsegmented ova are deposited in the feces; they are barrel-shaped and have a double shell, the outer one usually stained brown by bile. The inner, thick, translucent capsule has a knoblike protrusion at each end, somewhat like a bottle stopper. Segmentation occurs outside the body of the host and is a protracted process requiring from two weeks to many months. The ova are resistant, except to desiccation, and have been reported to retain their infectivity for five years. There is no intermediate host. Larvae



(*Left*) Ovum of *Trichuris trichiura*. (*Right*) Ovum of *Enterobius vermicularis*. (Photomicrographs × 200.)

hatch from ingested ova and become attached to the mucosa without migration; the parasite matures in about three months.

Enterobius Vermicularis. The common pinworm, threadworm, or seatworm is cosmopolitan in its distribution. It is the most common parasitic helminth in the United States, being found more frequently in children than in adults. The female is considerably larger than the male, with the vulva in the anterior third of the body. These worms have a clear, bulbous projection shaped like the mouthpiece of a Turkish pipe surrounding the three-lipped anterior extremity. There is a well-marked bulb which forms the posterior portion of the esophagus.

The eggs have a clear, transparent, doubly-contoured shell, flattened on one side and convex on the other (plano-convex). They contain a coiled embryo when deposited by the female and require only a few hours outside the body for development of the infective larval stage. The ingested eggs hatch and the larvae pass down the small intestine. They develop into adults on their way and attach themselves to the mucosa. The females become filled with eggs and then wander to the rectum to work their way out the anus and deposit the ova on the skin of the perineum. This life cycle requires about two months.

The presence of the gravid female produces marked itching, more pronounced at night, and the scratching so induced causes the eggs to be widely spread about the region of the anus. The fingers become contaminated with ova which may be carried to the mouth and cause a fresh infection. The possibility that eggs may be air-borne has been demonstrated. No intermediate host is necessary. The infection dies out spontaneously in a few months if reinfection is avoided but this is not easily accomplished. Ova are rarely found in the feces but may be found in scrapings from the skin about the anus or under the fingernails.

Physaloptera Caucasica. This is normally a parasite of monkeys and has been reported in man in eastern and northern Africa where it is said to be fairly common. The mouth of the adult is provided with two large, equal, laterally placed lips, each having two papillae and armed with teeth. The male has a lance-shaped posterior extremity with two unequal spicules. The female has a pointed tail and a vulva opening in the anterior part of the body. The ovum has a thick, smooth shell and contains a larva when passed. These parasites are usually found in the stomach and small intestine, rarely in the liver. The life history is not known but possibly involves some coprophagous insect as the first intermediate host.

Gnathostoma Spinigerum. *Gnathostoma spinigerum* has been found to occur in the larval stage in man in some parts of the Orient, chiefly Siam. The adult of this species is normally parasitic in the stomach wall of wild and domestic cats, forming hard cystic tumors 1 inch in diameter. In the life cycle, immature ova pass out with the feces and reach water. Here they mature and the larvae which hatch enter a microcrustacean (*Cyclops*). After further development and metamorphosis into a second larval stage within the cyclops, they must be ingested with their host by a frog, fish, or snake. The cat is infected when it devours the flesh of the second intermediate host containing an encysted larva. The parasite is not adapted to man, and in most cases immature worms which have "lost their way" have been found wandering in the subcutaneous tissues or skin abscesses. Human infection is probably caused by eating infected fish raw or insufficiently cooked. Sen and Ghose (1945) report a recent case in which this parasite was taken from the eye of a patient who they believe acquired the infection by accidentally ingesting an infected cyclops.

Clinical Illness. The symptoms and pathologic lesions produced by the intestinal nematodes, while primarily gastrointestinal in nature, are not confined to the alimentary tract. Those species which have a migration in their life cycles cause injury along the route taken by the larvae. The severity of symptoms is often related to the number of worms present, but many worms may cause no complaints and a single one may at times lead to death. In general, the symptoms attributed to these nematodes and the potentialities of such infections are often exaggerated. Since there is no multiplication within man, the pathologic lesions are roughly proportional to the number of ova ingested or of larvae penetrating the skin.

The most serious difficulties encountered with infections by these helminths are those of a mechanical nature. A group of pinworms may cause obstructive symptoms in the appendix; a single ascarid may likewise block the appendix; or several in a mass may obstruct the lumen of the bowel. An ascarid may enter the biliary or pancreatic ducts or perforate the intestinal wall. Biliary ascariasis may be very serious (Yang and Laube, 1946). Less serious, but no less objectionable, is the migration of adult *Ascaris* up the esophagus and out the nose or mouth. In an interesting study of clinical ascariasis, Swartzwelder (1946) reported six deaths in 202 cases. *Enterobius* may enter the urethra and vagina or even reach the fallopian tubes and has been found invading the tissues of the gut (Bylmer, 1946). These adults may carry secondary bacterial infections to such abnormal sites. Mechanical injury to the wall of the bowel may result from infection by those species which attach themselves to or invade the mucosa. All larval forms which migrate through the lungs produce some degree of pulmonary damage. Such symptoms may be mild or severe, depending upon the number of larvae and the presence of pre-existing pulmonary disease. The penetration of the skin by filariform larvae of *Strongyloides* and the hookworms may produce intense itching and skin lesions. Usually known as "ground itch" in hookworm and "creeping eruption" in infections by those species normally parasitic in dogs and cats, these lesions subside as the larvae pass on or die. Hartz (1946) reported an autopsy of a case of strongyloidiasis showing autoinfection in the colon.

In addition to the blood consumed by the hookworms, there may be free bleeding. As a result of the combined blood loss—perhaps of secondary bacterial invasion and the possible secretion of a toxin—severe anemia, weakness, general debility, and chronic ill health may develop with retardation of infected children. Hookworm infections constitute a major public health problem in many infested areas; over 90 per cent of the population may be infected. Recent studies have shown the great importance of a deficient diet in the production of anemia, often relieved by the administration of iron without elimination of the parasites. As a rule it probably requires 500 worms and several months to produce severe symptoms. However, much depends upon the resistance of the individual; it is believed that as few as 25 worms may cause definite disturbances in some persons. Steubenbord and Allen (1946) reported two fatal cases of ankylostomiasis from Guam in infants under one year of age. On the beneficial side, Duovir and

Brumpt (1944–45) have treated polycythemia with ankylostomiasis. Heavy infections with *Trichuris* have been shown to be very dangerous (Getz, 1945; Whittier et al., 1946).

Eosinophilia may be encountered in infection by any of these nematodes but is not invariably present. Allergic phenomena are most commonly seen in ascariasis. Loeffler's syndrome or tropical eosinophilia may be partly caused by *Ascaris* (Zweifel, 1944; Cardis, 1945) or hookworm (Wright and Gold, 1946) infections.

Diagnosis. The laboratory diagnosis of intestinal nematodes is most practically made by finding and identifying the characteristic ova or larvae, rarely adults, in the feces. Occasionally the urine or sputum may be examined. The ova of *Ancylostoma, Necator, Ascaris, Trichuris, Ternidens, Physaloptera,* and *Trichostrongylus* will be found in the feces; rarely *Enterobius* eggs will also be found, and ova of *Strongyloides* may appear after brisk purgation or in severe diarrhea. The feces should be examined by direct smear, flotation, and centrifugation. Sedimentation is rarely used for the nematode ova. Flotation methods offer the highest return by the simplest procedure, adequate in the majority of cases of infection with these helminths. Light infections, which tend to be overemphasized as a cause of illness, may be missed; this can be avoided by using a combination of flotation and centrifugation. However, these combined methods require much more apparatus and time. Specimens may be preserved and examined in the laboratory at a later date.

In using flotation methods it must be remembered that the ova are of different weights and the specific gravity of the solution should be adequate to lift them. The infertile *Ascaris* egg is the heaviest, requiring a solution of specific gravity of about 1.250; some infertile ova may be raised by specific gravities of 1.200. The critical specific gravity for hookworm is about 1.055, for whipworm about 1.150, and for fertile *Ascaris* about 1.130. Best yields are obtained with a specific gravity of at least 1.180 or higher.

The bile-stained ova of *Trichuris* and normal *Ascaris* are easily identified, but the transparent-shelled eggs of the hookworms, the decorticated ova of *Ascaris,* and the more rarely found ova of *Trichostrongylus, Enterobius, Ternidens,* and *Strongyloides* require careful differentiation. The criteria most commonly used are the size, shape, thickness of shell, and stage of development of the embryo. The two hookworms are practically indistinguishable by their ova, those of *Ancylostoma* usually being considered shorter; culture may be used and identification made from the larval stages. Stoll (1946) forcefully reiterates the point that *Ancylostoma duodenale* and *Necator americanus* cannot be distinguished by their ova.

Ova of two accidental parasites, *Heterodera marioni* and *Capillaria hepatica,* have been found in the feces of man several times. The eggs of *H. marioni,* a parasite of the roots and stems of edible plants, may be mistaken for those of hookworms or *Enterobius. Capillaria hepatica,* a parasite in the liver of rats and other rodents, has ova which are similar to those of *Trichuris.*

Larvae in feces are almost certainly *Strongyloides;* in old or constipated stools

Table 66

OVA OF INTESTINAL NEMATODES

Species	*Size (in microns)*	*Special Features*
Strongyloides stercoralis ..	50–58 x 30–34 Average 55 x 32	Oval, colorless, thin-shelled, developed to larval stage
Ancylostoma duodenale ...	56–60 x 35–40 Average 60 x 38	Oval, colorless, thin-shelled; poles bluntly rounded; usually developed to 4-celled stage
Necator americanus	64–76 x 35–40 Average 70 x 38	
Trichuris trichiura	50–54 x 22–23 Average 52 x 23	Barrel-shaped, brown, thick-shelled; mucoid plug at each pole; unsegmented
Ascaris lumbricoides	45–75 x 35–50 Average 60 x 45	Oval; outer wall mammillated, brown; inner wall thick, clear; unsegmented
Infertile	88–95 x 40–45	Irregular, rhomboidal; granular contents
Decorticated	43–68 x 33–48	With only clear, thick inner wall
Trichostrongylus species ..	70–105 x 35–55 Average 89 x 48	Oval, colorless, thin shell; one pole more pointed; usually morula stage
Ternidens deminutus	Average 80 x 50	Oval, colorless, thin shell; poles broadly rounded; developed beyond 4-cell stage
Enterobius vermicularis ...	50–60 x 20–30 Average 55 x 26	Oval, colorless, thin shell; flattened on one side; developed to larval stage
Physaloptera caucasica	Average 50 x 35	Oval, colorless, thick smooth shell; developed to larval stage
Capillaria hepatica	50–65 x 30–35	Barrel-shaped; brown, distinctly radially pitted, thick shell; unsegmented
Heterodera marioni	80–120 x 25–45	Bean-shaped, colorless, thin shell; air space at poles; developed to morula stage

the larval stages of the hookworms or *Trichostrongylus* may have hatched from the eggs, and in rare instances free-living coprophagous forms (*Rhabditis*) may contaminate the specimen. Methods used should include direct smears and centrifugation. Good results have also been obtained with the zinc sulfate flotation-centrifugation method, but flotation technics alone are usually unsatisfactory. Culture methods and the Baermann apparatus are also of definite value in diagnosing suspected infections where larvae may be found. Watson (1946) gives an excellent discussion of the differential characteristics of the ova and larvae of the hookworms, *Strongyloides* and *Trichostrongylus.*

Trichostrongylus and *Rhabditis* larvae resemble the hookworms in having buccal cavities which are considerably longer than the body width and small genital rudiments. The esophagus of *Rhabditis* is pseudo-rhabditiform, having a median swelling in addition to the terminal bulb. *Trichostrongylus* is distinguished by its long filamentous tail.

Table 67

RHABDITIFORM LARVAE OF INTESTINAL NEMATODES

Characters	*Strongyloides stercoralis*	*Ancylostoma and Necator*
Size	200–250μ (in stool)	250–300μ (when hatched)
Usually	225μ	275μ
Buccal cavity	10μ	20μ
Length	Shorter than body width	Longer than body width
Genital primordium	Large; about 35μ	Small; about 20μ
Tail tip	Short; bluntly pointed	Short; sharply pointed

When culture methods are used, or very rarely in the stools, filariform as well as the earlier larval stages may be found. The esophagus of the infective larva of the hookworms is not typically filariform; a more correct term is pseudo-filariform. The pseudo-filariform larva of *Trichostrongylus* has a tuberculated tail; there is no filariform larva in *Rhabditis*.

Table 68

FILARIFORM LARVAE OF INTESTINAL NEMATODES

Characters	*Strongyloides stercoralis*	*Ancylostoma and Necator*
Size	500–700μ	About 800μ
Genital primordium	Behind middle of body	Near middle of body
Esophagus		
Length	About ⅖ body length	About ¼ body length
Shape	Narrow throughout length	Slight posterior bulb
Tail tip	Minutely notched	Pointed

Although seldom necessary, *Ancylostoma duodenale, Necator americanus,* and the other hookworms can be differentiated by their filariform larvae. Those of *A. duodenale* have a slightly flattened head and a rather narrow esophagus without a definite constriction at the esophageal-intestinal junction; also the esophageal spears are inconspicuous and unequal. In *N. americanus* the head is smaller and more cylindrical and the esophagus is broader with a marked constriction where it joins the intestine; the esophageal spears are conspicuous and equal. The tail of *A. braziliense* is more slender than in the other two species.

For the diagnosis of *Enterobius* infection, the collection of ova or adult females from the perianal folds is the method of choice. The NIH swab is a time-tested technic which gives excellent results. The scotch-tape method is a more recently devised technic that has many advantages. Ova may also be collected from other regions of the body, from clothing, and from furniture in homes containing infected persons by using the transparent tape; they may also be recovered from fingernail scrapings. In about one-half the cases of ascariasis and one-sixth of trichuriasis, ova may be collected from the perianal region. Watson and MacKeith

(1946) have summarized the various technics used in obtaining anal smears.

Adult intestinal nematodes are sometimes evacuated spontaneously or after treatment and may then be identified by their anatomic features (see key, Chapter 24). The smaller worms may require special methods of recovery. Quite often *Enterobius* females may be scraped from the perineum. *Ascaris* adults tend to migrate and have been taken from the nostrils and mouth in numerous cases.

Larvae of species which migrate through the lungs and respiratory passages (*Strongyloides,* the hookworms, *Ascaris*) may very rarely be recovered from the sputum. The direct smear and centrifugation may be employed in demonstrating them. In a few rare instances larvae or eggs have been found in the urine. Some workers have reported greater success in finding *Strongyloides* larvae by using a duodenal tube, suggesting that they are destroyed or digested as they pass out with the feces; other statistics show only a slight increase in positive findings by this method. Duodenal drainage is not recommended except in cases where no evidence of the parasite is found in the feces and there is strong suspicion of *Strongyloides* infection. However Denhof (1947) found duodenal intubation a highly successful diagnostic procedure. Lapev (1945) recovered both larval and mature forms of *Strongyloides* from the sputum of a patient.

It may be desirable to recover intestinal roundworm larvae or ova from the soil. The Baermann apparatus is an ingenious and practical device for obtaining the larvae of hookworms, *Strongyloides,* and, it should be emphasized, free-living nematodes. Other methods may be used to recover eggs from the soil.

Serologic methods for the diagnosis of intestinal nematode infections exist but are of little practical significance.

Trichinella Spiralis

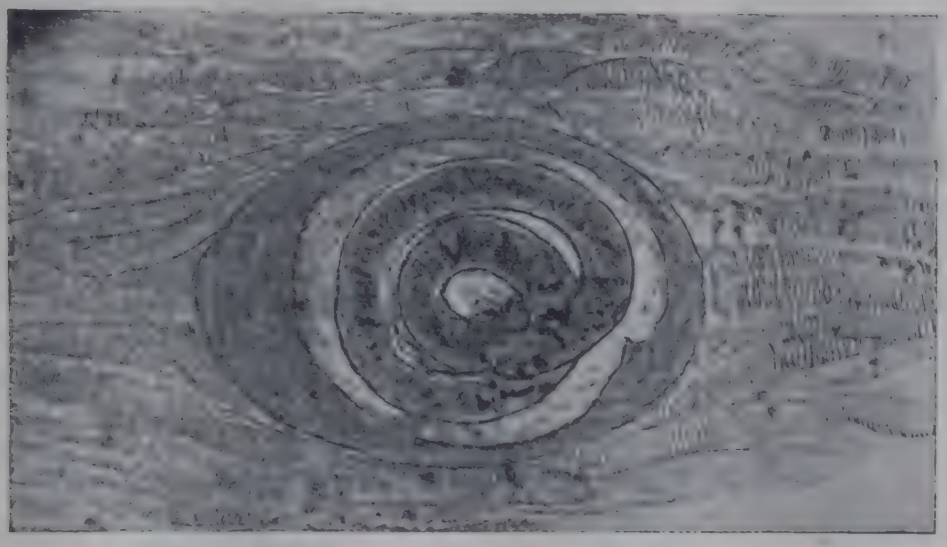
The trichina worm which is the cause of trichinosis has a world-wide distribution. As the source of human infection is infected pork, the disease is practically unknown in people of the Mohammedan and Jewish faiths. The incidence of infection in the United States has been reported as about 17 per cent, determined by postmortem examination of diaphragms. The majority of infections are unrecognized.

Morphology. The adults are small white worms just visible to the unaided eye, the male being 1.5 mm. long and the female about 3.9 mm. The stout posterior half of the male is filled mostly with testes. The posteriorly located cloaca is eversible, being guarded by two cone-shaped papillae which clasp the female in copulation. The rounded posterior half of the female contains the ovary and becomes distended as the eggs develop. The vulva is anterior, a location which facilitates the extrusion of larvae into the tissues by the burrowing female. The larvae are about 100μ long and 6μ in diameter, a size which permits transit through the capillaries of the host.

Life History. Two hosts are required for the completion of the life cycle. While the larvae encyst in the same individual that harbors the adults, they are liberated only when the cysts are ingested by another host. The infection is

normally propagated in nature by black and brown rats which are cannibalistic; pigs, wild boars, and other mammals which eat flesh become secondarily infected. Hogs constitute the principal reservoir for human infection, the relation between rats, swine, and man being indicated in the drawing of the life cycle. Man has contributed to his own infections by feeding raw pork scraps in garbage to hogs.

When viable cysts are swallowed the larvae are liberated in the intestine and develop into mature males and females within a few days. The female is ovoviviparous, depositing larvae and not eggs; a single female may give birth to at least 1500 larvae over a period of six weeks. Most of these reach the portal or lymphatic systems and ultimately the heart, being distributed from there by the systemic circulation. They may become lodged in various tissues of the body but are capable of further development and encapsulation in only voluntary



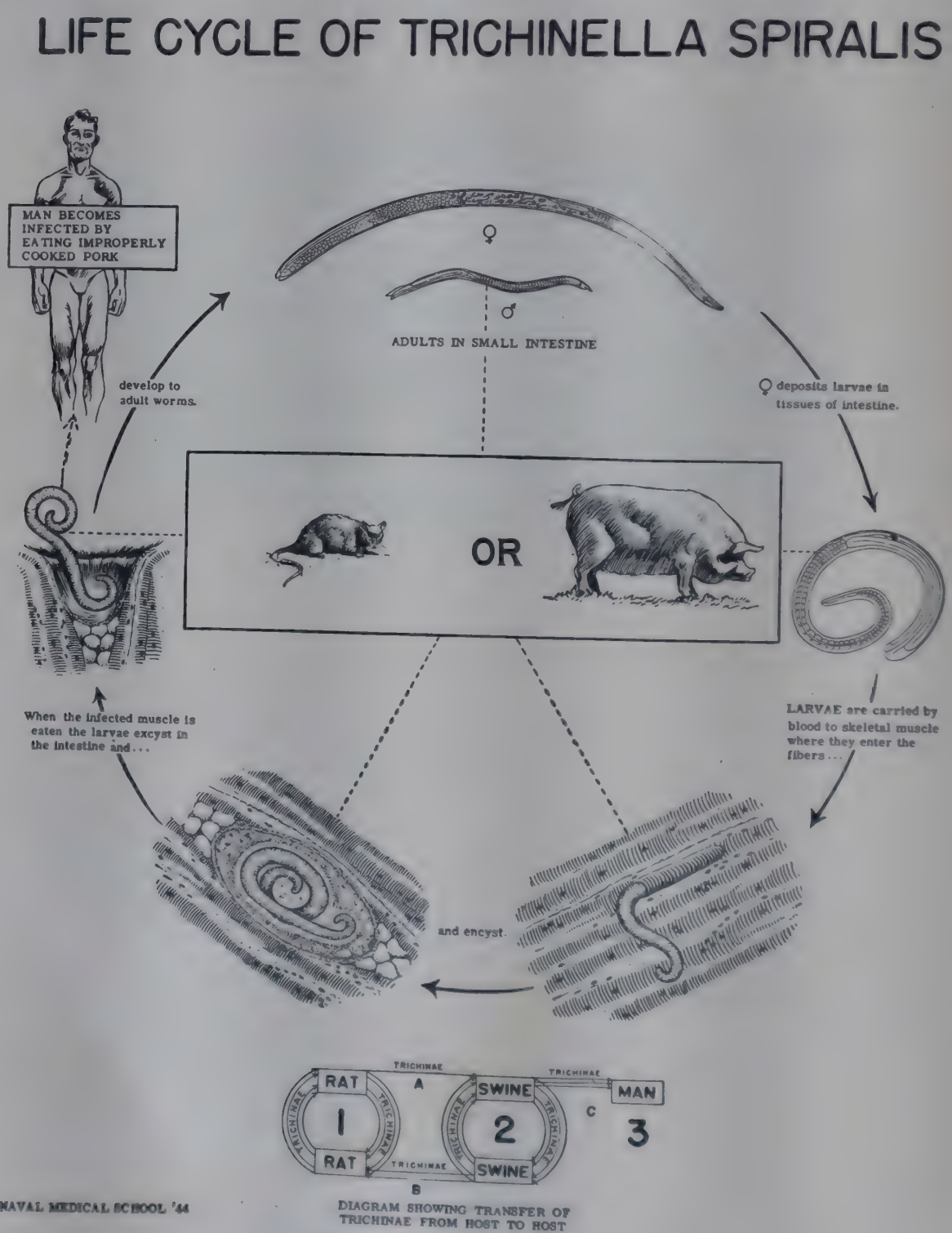
Larva of *Trichinella spiralis* encysted in striated muscle. (Photomicrograph × 75.)

(skeletal) muscle. The greatest invasion occurs in those muscles with the richest blood supply—the diaphragm and intercostal, laryngeal, tongue, and eye muscles. The larva is provided with a spear, a movable boring apparatus at the anterior end, by which it is aided in penetrating muscle tissue. It comes to lie along the long axis of the muscle fiber, grows to about 1 mm. in length in 10 to 14 days, and assumes the spiral form of the encysted parasite. The capsule is an ellipsoidal sheath with blunt ends resulting from the infiltration of protective cells of the host. Here the larva remains viable for a long time (some for even 10 to 20 years), eventually dying and the cyst becoming calcified.

Prophylaxis. Prevention depends on adequately cooking pork or, rarely, the flesh of other carnivores. Refrigeration of meat for 20 days at 5° F. (—15° C.), for 10 days at —10° F. or for 6 days at —20° F. also kills the parasites. Meat inspection is not sufficient; in Germany one-third of the cases of trichinosis were traced to inspected meat. Inspection in the United States does not attempt to detect the parasite; pork that will be used for products which may be consumed without cooking (summer sausage) is treated by refrigeration in approved packing plants. Uncooked garbage should not be fed to hogs. The slide flocculation test

(Suessenguth and Kline, 1944) for the detection of infected swine offers great promise but at present is not accurate enough for use.

Clinical Illness. The severity of the disease depends primarily upon the number of encysted larvae swallowed; in severe epidemics the fatality often reaches 30 per cent and death may come quickly after heavy infections. During the first few days while the ingested larvae are developing, there is often a gastroenteritis. Purging may then be beneficial by ridding the intestine of some of the parasites before they have burrowed into the mucosa. In severe cases there is a high fever, marked intoxication, and prostration during the second and third week, as in



Life cycle of *Trichinella spiralis*.

typhoid fever. The larvae are then present in the circulating blood. There is often transient edema, especially about the face and eyes. The blood count usually shows a high leukocytosis with an eosinophilia of 5 to 90 per cent, beginning as early as the tenth day, often rising rapidly, and reaching a peak during the third or fourth week; in heavy infections, usually those that are fatal, eosinophilia may not develop.

From the tenth day on the larvae migrate into the muscles and become encysted. The invaded muscle fibers rapidly undergo degeneration. This is associated with severe pain, stiffness, and disability; there is often painful labored breathing or laryngitis with cough and occasionally hemoptysis. Gould (1945) has given a detailed summary of all aspects of trichinosis.

Diagnosis. An early laboratory diagnosis is extremely difficult and rarely made. During the first few days after infection, adult worms may be found occasionally by sedimentation of the feces. After the sixth day and until the end of the migration period, larvae may be demonstrated in the blood. This is of value only in heavy infections, yielding about two larvae per milliliter of blood. Larvae may also be recovered from the spinal fluid. Biopsy for encysted forms is most practically done upon the deltoid, biceps, or gastrocnemius muscles. Compression and digestion methods are used; care must be exercised so as not to be misled by old infections.

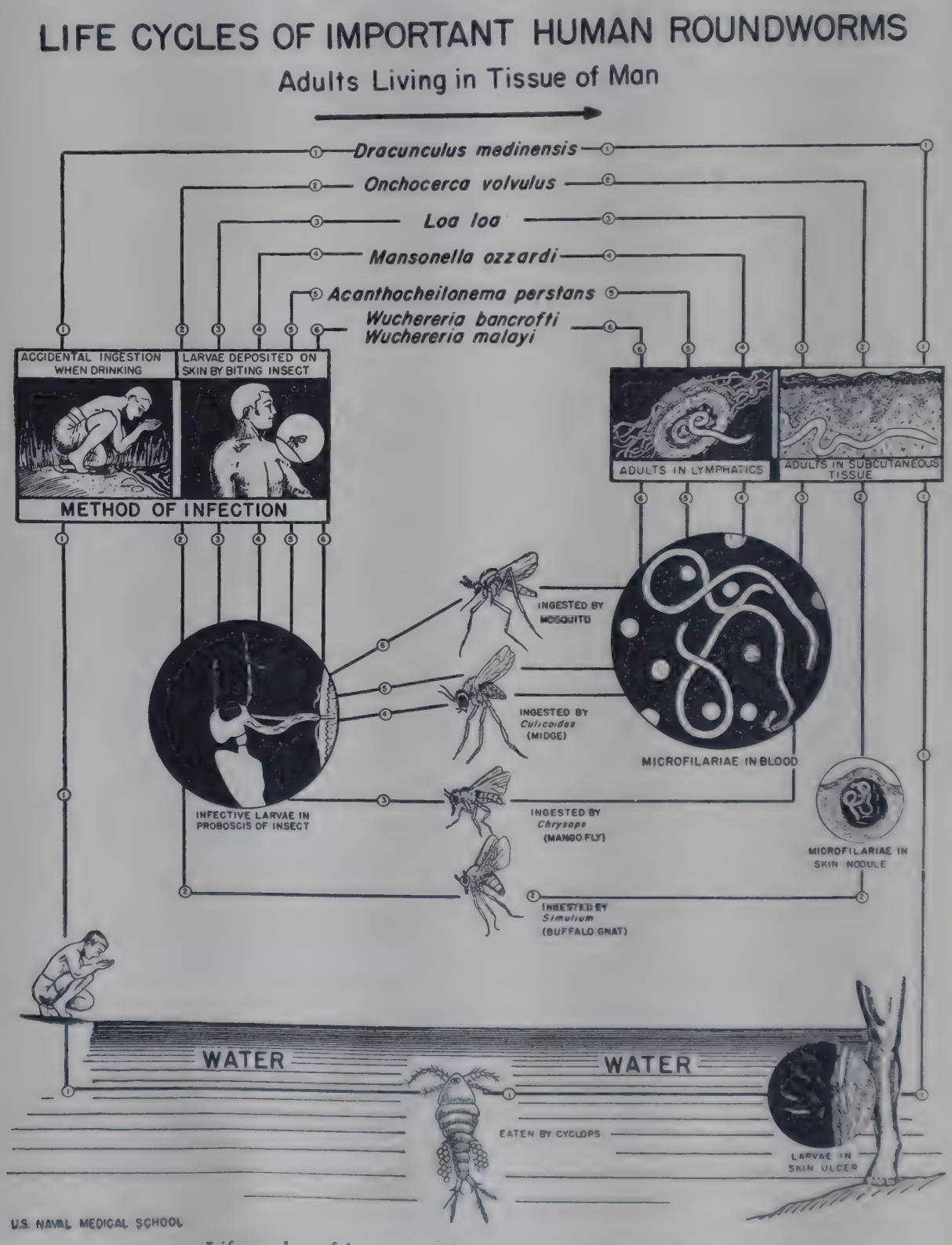
Serologic and allied tests are not sufficiently specific to be more than an adjunct to the clinical diagnosis. Reaction to the skin test becomes positive earliest but the test is of no value before the twenty-first day; very light and very heavy infections may not be detected by this method. False positive reactions occasionally occur as do nonspecific reactions due to diluting fluid. After the fourth week, precipitation and complement-fixation tests become useful. The specificity of precipitation is about the same as that of the skin test. Although complement fixation gives greater accuracy in diagnosis it is seldom practicable to use it because of the complexity of the procedure. Roth (1945, 1946) has recently devised tests for trichinosis using living larvae and immune serum which may prove valuable in diagnosis.

Postmortem examination includes digestion, compression, and sectioning methods. Small bits of diaphragm give the highest percentage of positive findings, but other striated muscles should also be examined.

Somatic Nematodes

The somatic or tissue roundworms include the filariae and the guinea worm—species in which the adults live in the tissues of man, other than those of the gastrointestinal tract. All of these forms require an arthropod intermediate host in which the larvae develop but do not multiply. The two species of *Wuchereria,* etiologic agents of the disease commonly referred to as *filariasis,* utilize mosquitoes as the intermediate host and vector; these parasites are widely distributed and of considerable importance. The other somatic nematodes are of less significance, being confined to more restricted areas where the reservoir hosts and the appropriate intermediate hosts are intimately associated.

Life Histories. The adult filarial worms (*filariae*) and the guinea worm live in the lymphatics or subcutaneous tissues where, instead of laying eggs, they give birth to embryos (*microfilariae* and *larvae*). The adult guinea worm deposits her larvae in small cutaneous ulcers; when these ulcers burst on contact with water the larvae are freed and may be ingested by the intermediate host, a microcrustacean—*Cyclops*. The microfilariae, on the other hand, migrate to the peripheral blood vessels or subcutaneous lymph spaces where they are picked up by the intermediate host, a biting dipterous insect, with its blood meal. Only



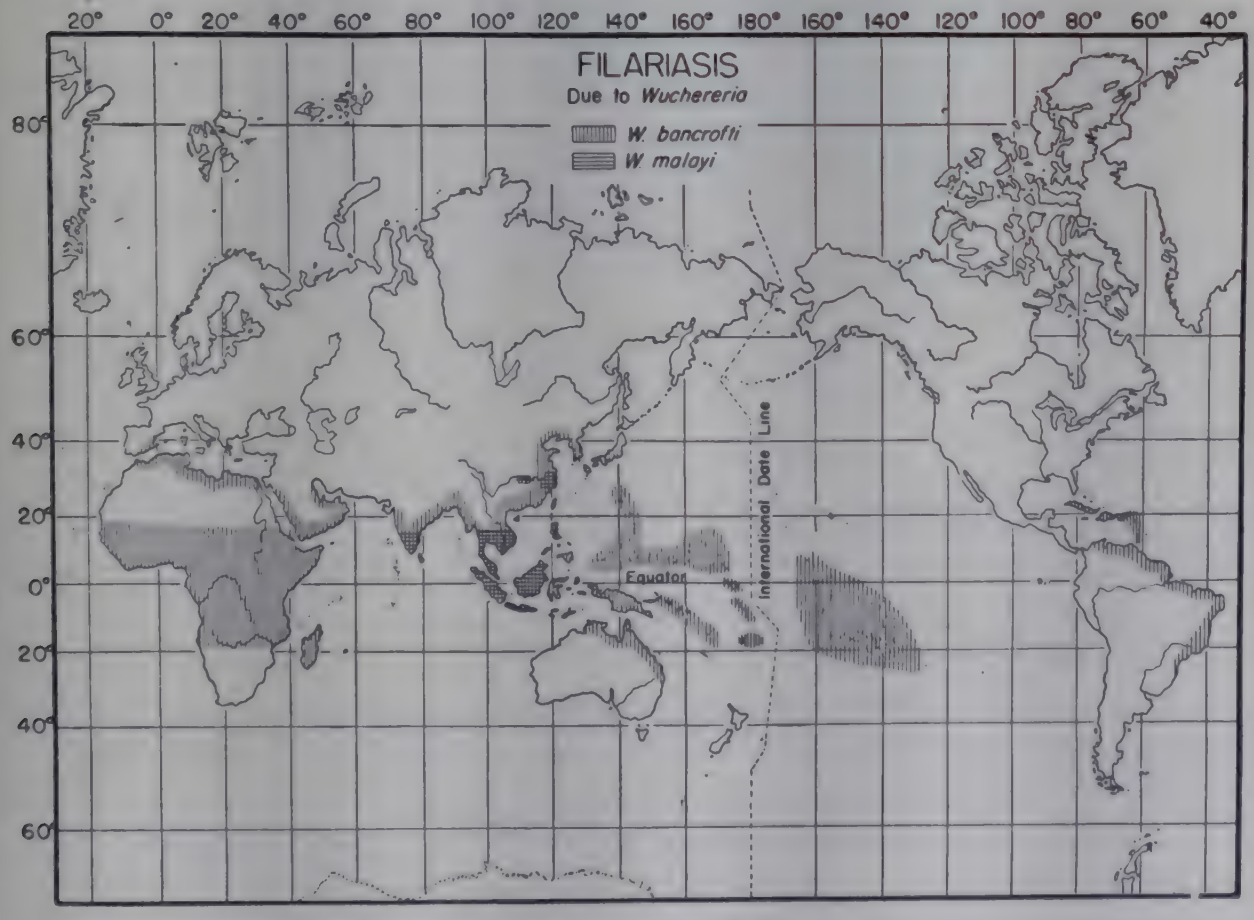
Life cycles of important human somatic roundworms.

certain insects are capable of acting as the intermediate host for each species of filarial worm.

The microfilariae have a fairly uniform development within the insect host. The sheath, if present, is shed and the microfilaria penetrates the gut wall and migrates to the thoracic muscles where at least two molts occur. The infective larva then passes to the proboscis and remains there waiting for the opportunity to be transferred to man. The larva of the guinea worm undergoes a comparable development to an infective stage within the cyclops but remains in the body cavity of this host.

Man acquires the guinea worm when he ingests infected microcrustaceans in drinking water. The infective filarial larvae leave the proboscis while the insect feeds and penetrate the skin of man. The period from the entrance of the larvae until the mature female worms begin producing embryos is incompletely known for all species. Man is the only reservoir host of the filarial worms; man and fur-bearing mammals are definitive hosts of identical or closely related species of the guinea worm.

Prophylaxis. The prevention of infection by filarial worms is accomplished by avoiding close contact with the various insect vectors which have become infected from the native reservoir or by control of the insects involved (see Medical Entomology). Because of the large number of species which may transmit *Wuchereria,* the mosquito vector must be determined for each area and those control measures employed which will be most effective against that species. Bed



Geographic distribution of filariasis.

nets, repellents, insecticides, and screened quarters must be utilized. Since many of the vectors are day-biting insects, control measures must be extended to cover daylight hours.

The proper care and selection of drinking water will prevent infection with the guinea worm.

Species: WUCHERERIA BANCROFTI AND W. MALAYI. Infection by one or both of these species is endemic throughout the more moist and warm tropic regions of the world. The so-called "filarial belt" extends around the earth at the equator and encompasses an area reaching from approximately 30° South to 35° North latitude. In general, filariasis can be said to be a disease of the coastal plains and inland waterways. *Wuchereria bancrofti* is found throughout the entire filarial belt; *W. malayi* is endemic in southern China, Indo-China, Malaya, southern India, and the East Indies. Although the latter has been introduced into many areas of the southwest Pacific within recent years by the importation of laborers from continental Asia, infection is probably not endemic in these areas. Filariasis is spotty in its distribution. Frequently it has been possible to show a very high incidence of infection in one locality, a low incidence in one near-by, and complete absence in still another. This is true for the entire filarial belt and for islands within an island group as well as for different locations on the same island.



Microfilaria of *Wuchereria bancrofti*, showing sheath. (Photomicrograph × 250.)

MORPHOLOGY. The adult worm is white and threadlike with a smooth cuticle; the head is provided with two rows of small papillae. The small, unarmed oral opening leads directly into the esophagus, there being no mouth cavity. The females of the two species are indistinguishable, but *W. bancrofti* is usually larger, about 80 mm. as compared with 50 mm. for *W. malayi*. The males of *W. bancrofti* are about 40 mm. in length and have a large number of anal papillae and stout spicules with distinct transverse striations; those of *W. malayi* are smaller, about 25 mm., and have two large and two small pairs of papillae and more delicate spicules which lack transverse striations. Differentiation of these two species from each other and from other species of filarial worms depends mainly upon the characters of the microfilariae. The important features are given in the diagnostic chart and in the key (Chapter 24).

LIFE CYCLE AND TRANSMISSION. The adult parasites live in the larger lymph channels of man. The fertilized female passes the sheathed embryos (microfilariae) indirectly into the blood stream where they are picked up by the feeding mosquito. More than 50 species of mosquitoes, about equally divided between anophelines and culicines, are known to be capable of transmitting *W. bancrofti;* about half of these are natural vectors and the remainder have developed larvae to the infective stage experimentally. *Culex quinquefasciatus* and *Aedes pseudoscutellaris* are probably the more common vectors. Only about a dozen species are known to transmit *W. malayi,* both anophelines and culicines being involved; *Mansonia*



Infective filarial larva of *Wuchereria bancrofti* extending from labium of a mosquito. (Photomicrograph × 100.)

species are the usual vectors. There is a high mortality in recently infected mosquitoes.

Depending upon the humidity and temperature, the larvae require from 6 to 20 or more days to complete their developmental cycle in the mosquito host; the average is about 14 days. Development takes place in the thoracic muscles and at least two molts occur during this phase of the cycle. Following the second molt the larvae become increasingly active and soon leave the thorax to migrate to all parts of the body; some find their way to the labium of the mosquito where they await transfer to the next human host. By the time the infected mosquito is about half through feeding the membrane at the tip of the labium is ruptured and the infective stage larvae are spilled on the skin along with a drop of plasmalike fluid from the labium. They disappear into the skin as the fluid dries. Pratt and Newton (1946) have shown that infective larvae will escape from the mosquito

while the latter feeds on sugar water, or when the proboscis ruptures spontaneously, probably as a result of being too tightly packed. Nothing is known of the route the larvae take in arriving at their definitive site within the human host; nothing is known concerning the development of the larvae into adults. Microfilariae are not commonly found in the blood of native children under five years of age although they have been demonstrated in those as young as 18 months.

Each infective larva represents a single microfilaria that has succeeded in developing in the mosquito, and each adult worm represents an infective larva that has been successfully transferred to man. A single bite by an infected mosquito is not likely to result in clinical evidence of infection; the majority of clinical cases and those showing microfilariae circulating in the blood come from areas where repeated infections over many years have occurred. Even when massive infection has occurred in a short period of time, the slow progress of the disease in man suggests that military personnel returning to the United States will rarely harbor microfilariae in the blood in sufficient numbers for these to be demonstrated or for a vector to pick them up. The maintenance and propagation of this disease, probably more than other mosquito-borne infections, depends upon a close and free association between infected persons and the vector. The local native population of an endemic area is the only source of filariasis.

The incidence of infection in the native reservoir ranges from a fraction of 1 per cent to approximately 100 per cent. Considerable survey work has been done in regard to both incidence and transmission in many areas of known high endemicity; other areas have been partially studied or neglected altogether. A true picture of conditions existing in many localities will not be available for many years to come. While the incidence of Malayan filariasis often equals or exceeds Bancroftian in the same area, the number of circulating microfilariae is much higher in *W. bancrofti* infections. On the other hand, the incidence of elephantiasis in some areas is much higher with Malayan infections.

Although most authorities emphasize the periodic nocturnal appearance of Bancroftian microfilariae in the peripheral blood, the form or strain of *W. bancrofti* of greatest interest in the United States at the present time is practically nonperiodic. Personnel of the armed forces with filariasis have acquired their infections almost exclusively in the Samoan and nearby islands. If large numbers of Samoans are examined, the so-called nonperiodic type is found to be a highly modified nocturnal form, and in some individuals there is a diurnal rhythm. *W. malayi* presents a periodicity which is nocturnal but not nearly so sharply marked as that of *W. bancrofti* in the East Indian, African, and Puerto Rican areas. The causes of microfilarial periodicity are sharply debated; one important factor appears to be the biting habits of the mosquito vectors.

The location of the noncirculating microfilariae is not known. The former reports by Manson-Bahr that they were in the lungs have been partly confirmed by their recovery at autopsy of a native on Saipan (Seronde, personal communication, 1945); Japanese physicians on Okinawa were reported to prefer lung puncture as giving more positive diagnoses (Cheever, personal communication, 1945).

Onchocerca Volvulus. Infection by this species is common in scattered areas of central Africa, especially the Belgian Congo, and in the mountainous regions of western Guatemala and southern Mexico where it was described as *O. caecutiens* Brumpt, the "blinding filaria." The adult worms are found in the subcutaneous lymphatics of man, usually enclosed in fibrocystic nodules 2 to 30 mm. in diameter. Several worms, male and female, may be found in each nodule, coiled up and imprisoned in a mesh of connective tissue. The female worm is about 35 cm. long and 0.4 mm. in diameter, the male 3 to 4 cm. by 0.15 mm. The cuticle is striated. Microfilariae are very numerous in the cystic fluid and in the lymphatics of the skin but rarely enter the blood; their size is about the same as *W. bancrofti.*

The intermediate host in Africa is the jinja fly, *Simulium damnosum,* and probably *S. neavei;* in the Western Hemisphere, *Eusimulium avidum, E. ochraceum,* and *E. mooseri.* These blood-sucking insects are commonly called black flies or buffalo gnats; the occurrence of related species in the southwestern United States suggests the possibility that the disease might become established if introduced into this region. Wanson et al. (1945) report that the development in the insect requires 6 to 7 days.

Loa Loa. The eye worm is the causative agent of Calabar or fugitive swellings. It occurs commonly in western and central Africa, especially in the Congo region. The male is about 30 $\times$ 0.4 mm., the female being longer, up to 70 $\times$ 0.5 mm. The cuticle is studded with numerous rounded bosses about 15μ in diameter and 10μ in elevation. The anterior extremity is shaped like a truncated cone. There are two short, unequal spicules in the male; the vulva in the female opens near the anterior end.

The microfilariae closely resemble those of *W. bancrofti* but can usually be distinguished in stained preparations. They have a definite diurnal periodicity. The intermediate hosts are the deer or mango flies, *Chrysops dimidiata* and *C. silacea,* in which a development occurs similar to that of *Wuchereria* in the mosquito.

Mansonella Ozzardi. *Mansonella ozzardi* is a filarial worm of little or no importance in clinical aspects. Its distribution is limited to the Western Hemisphere, being found in Central and South America and the Caribbean region. The adults live in the body cavities of man. The adult female is about 70 mm. in length; the complete male is unknown. The smooth cuticle and a pair of fleshy posterior lobes are characteristic. The microfilariae are nonperiodic in their appearance in the peripheral blood. The intermediate host is a midge, *Culicoides furens,* and possibly other species.

Acanthocheilonema Perstans. *Acanthocheilonema perstans* is found most commonly in tropical Africa, South America, and the Caribbean region. The adult worms are found chiefly in the mesenteries and the retroperitoneal connective tissue. They resemble those of the preceding species in having a smooth body, but have an incurved tail, the extremity of which has two triangular appendages which give it a bifid appearance. The microfilariae are small and show no periodicity. The intermediate host is a midge, *Culicoides austeni* or *C. grahami.*

Dracunculus Medinensis. The guinea or Medina worm is common in parts of

India, Africa, and Arabia and has become indigenous in parts of the West Indies, Brazil, and Guiana. A parasite morphologically identical with *D. medinensis* has been found in various carnivores (fox, raccoon, mink) in the United States and appears to be established in this country, but no infections in man have been reported. Dogs may be infected naturally and artificially.

MORPHOLOGY. The female is threadlike and cylindrical, 1.6 mm. in diameter, and may reach a length of 1.0 meter. She lives in the subcutaneous tissue and intermuscular connective tissue, particularly that of the lower extremities. The tip of the tail is bent, forming a sort of anchoring hook. The mouth is terminal. The uterus is a continuous tube occupying the greater part of the body and filled with sharp-tailed, transversely striated, unsheathed embryos which measure 600 × 20μ. The males obtained by Leiper from experimentally infected monkeys were less than 2.5 cm. in length.

LIFE CYCLE AND TRANSMISSION. When the female is mature and fecundated she migrates to the subcutaneous tissues and the head penetrates into the dermis to produce a cutaneous blister. The larvae are discharged by a gradual prolapse of the uterus through the ruptured head; the blister breaks when immersed in water, and the larvae escape to swim about until swallowed by *Cyclops quadricornis* or related species. In this intermediate host the parasites develop for four to six weeks, reaching a length of 1 mm.; they then remain dormant until the cyclops is swallowed by the definitive host in contaminated drinking water. Leiper has shown that hydrochloric acid in the concentration present in the gastric juice kills the cyclops but makes the *Dracunculus* larvae very active; they doubtless bore through the stomach wall and make their way to connective tissue. Development to maturity in man is believed to take about one year.

Clinical Illness: FILARIASIS. Of the several diseases produced by the somatic nematodes, only that caused by the species of *Wuchereria* is of major importance. Filariasis in natives appears to be very different from the disease seen in Americans who have acquired the infection. The clinical picture commonly described emphasizes the late sequelae—elephantiasis of arms, breasts, and genitals; chyluria; chylous ascites and diarrhea. Such sequelae result from multiple infections and exposure over many years and affect only a small proportion of the total population infected. Filariasis as encountered in Americans who have had relatively light exposure over short periods of time produces early manifestations rather than late.

The incubation period is subject to considerable variation. In one group of 550 men infected in Samoa, symptoms were noted as early as one month in one case while three developed their first evidence of infection 21 months after exposure; the average for the group was nine months. The earliest symptoms are recurrent lymphadenitis and lymphangitis, with or without slight febrile response, nausea, and malaise; there may be allergic phenomena such as chilliness, urticaria, periorbital edema, and conjunctivitis. The lymphangitis, usually preceded by swelling and marked tenderness of regional lymph glands, is described as descending, centrifugal, or retrograde in contrast to the common inflammatory type. Funiculitis, with or without epididymitis, acute hydrocele, orchitis, or edema of the scrotal

skin, is often the first manifestation when the parasites are located in pelvic or inguinal sites. Rose et al. (1945) believe these attacks of lymphangitis are allergic rather than streptococcal in nature.

At first these episodes endure for a few days and then subside; in the intervals it is not always possible to detect physical signs that can be attributed directly to the specific infection. Approximately 50 per cent of the cases have followed this pattern. However, a series of recurrences may follow with involvement of the same or new lymphatic areas. Subjective evidence may precede the objective signs and seems to increase in severity with recurrences. As months pass, symptoms continue although no further exposure to infection has occurred. The acute episodes may disappear leaving complaints of aching and weakness of muscles, dragging pain in the testicles, paresthesias, and general malaise and depression; there may be a diffuse thickening or nodule formation along the spermatic cords, a lymph varix, or a hydrocele. At the end of the third year the significant symptoms have disappeared in nearly all cases.

The late manifestations, which up to the present time have been encountered only in natives, are due to lymph stasis from obstruction of the larger lymph vessels and accompanying tissue changes. In the vast majority of natives with *Wuchereria,* the only positive evidence of infection is the presence of circulating microfilariae.

Onchocerciasis. Clinical evidence of infection with *Onchocerca volvulus* is manifested by the subcutaneous nodules. In Africa these are usually over the ribs, in the axillae or popliteal spaces, or on the elbows; in America, about the head. Cases of elephantiasis have been attributed to *Onchocerca volvulus* in the Congo. The microfilariae cause irritation of the skin in varying degree and appear able to spread for a considerable distance from the nodules. In chronic cases and especially those with nodules about the head, they may penetrate into the tissues of the eye and cause intense irritation, lacrimation, photophobia, a punctate keratitis with iritis and uveitis, a choroiditis, and possibly a retrobulbar neuritis. Microfilariae have been reported in the optic nerve itself and blindness may occur. Earlier observations indicated that ocular manifestations subsided promptly after excision of all nodules, thus cutting off the supply of microfilariae; subsequent reports have not confirmed this. First reported by Robles (1916), this condition has been described at length in the monograph of Strong et al. (1934). Although involvement of the eye has been considered peculiar to the American infections, Hisette (1923) has described identical lesions in more than half the population of certain stricken villages in the Congo. Bryant (1935) has reported onchocerciasis in the Sudan and believes it to be the major cause of "Sudan blindness." Ridley (1945) has emphasized the seriousness of this disease when it affects the eye.

Loiasis. Infection with *Loa loa* develops slowly; from three to six years may elapse before microfilariae appear in the blood. The adult worms living in the connective tissue wander extensively and may appear in superficial tissues, occasionally about the eyelid or under the conjunctiva where they cause more or less serious local irritation. They may cause areas of localized edema, particularly on

the hands and arms; these Calabar swellings are about the size of hen's eggs, appear abruptly, are painless, do not pit on pressure, and subside in about three days. They have been regarded as allergic phenomena. Microfilariae may not be demonstrable in the blood of infected persons, even those from which adult worms have been recovered.

Mansonella Ozzardi and Acanthocheilonema Perstans Infections. These produce few or no symptoms in man, although there are occasional reports of pathologic changes due to the parasites (Garratt, 1945).

Dracunculiasis. The guinea worm causes no symptoms during its development. Penetration of the dermis produces a painful ulcer accompanied by burning and itching and often by nausea, vomiting, diarrhea, urticaria, asthma, and fainting; such symptoms suggest an anaphylactic reaction.

Diagnosis. The diagnosis of filarial disease is usually made by finding and identifying the characteristic microfilariae; adults are rarely recovered. The microfilariae of all species except *Onchocerca* occur most commonly in the blood. Those of *Wuchereria bancrofti* and *W. malayi* may also be found in fluid aspirated from a hydrocele or an enlarged lymph node and rarely in the urine. *Onchocerca* microfilariae may be found in fluid aspirated from a nodule and in biopsied skin teased out in saline; they are rarely demonstrable in blood. Puig-Solanes et al. (1945) were able to diagnose 79.5 per cent of 92 cases of onchocerciasis by biopsy.

Living microfilariae may be easily seen in fresh blood smears examined by the low-power lens. They are continuously lashing and writhing, moving the red blood cells. Species identification is practically never possible on living unstained embryos; thick blood films as for malaria or concentration methods should be used. Preparations may be stained by Giemsa, methylene blue-eosin, or hematoxylin. Although more time-consuming and requiring more materials, the latter method results in slides which are more permanent and show the diagnostic features more clearly.

It must be emphasized that the microfilariae of *Wuchereria bancrofti* and *W. malayi* do not occur in early cases and may not be found in very late cases. It has rarely been possible to demonstrate circulating forms in Americans with early symptoms of filariasis; it is not expected that these cases will ever progress to the point where microfilariae will occur in more than rare instances. The accompanying figure (p. 625) shows almost all of the morphologic characters used in differentiation of the species.

If the species cannot be identified by the criteria given in Table 69, other features may be used; however, the clarity of morphologic landmarks depends upon good fixation and staining, and if the characters tabulated are indistinguishable it is unlikely that other anatomic features will be seen. Some of the more helpful points of microfilaria behavior and additional diagnostic characters are briefly discussed.

Periodicity. As mentioned previously, the microfilariae of *W. bancrofti* may show a marked or modified nocturnal periodicity, or even a diurnal rhythm, depending upon the geographic area involved; *W. malayi* usually shows a moderately

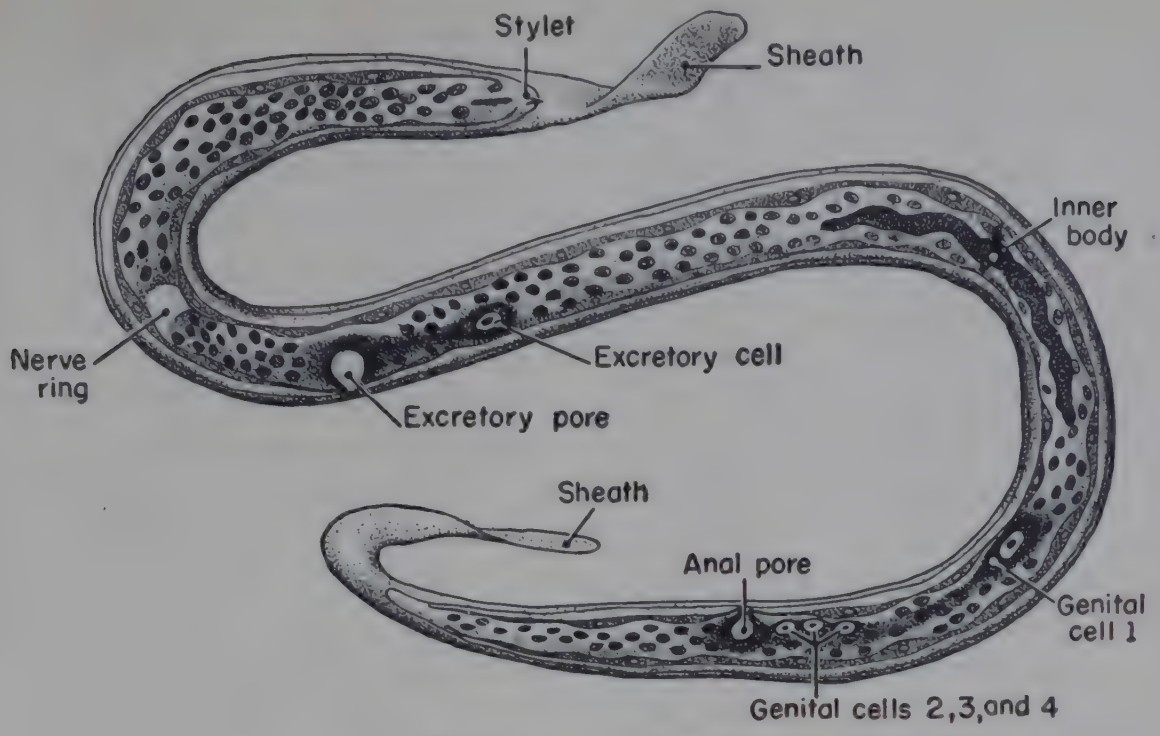


Diagram of a microfilaria.

marked nocturnal periodicity. *Loa loa* has a sharply marked diurnal rhythm and the other species no periodicity whatsoever. Blood should be taken for examination accordingly.

Anterior End of Microfilariae. This is of importance in *Loa loa* which has a more or less flattened head, the other species being rounded. The point at which the nuclei terminate in the cephalic end is also of help in distinguishing *Wuchereria bancrofti* from *W. malayi;* in the former the space without nuclei is about equal in length and width, while in the latter it is about twice as long as wide.

Tip of Tail. The shape of the tail may be helpful in differentiation. The tail is usually straight and tapers to a point in *W. bancrofti,* is sharply pointed with a

Table 69

Microfilariae

Species	*Length (in microns)*	*Appearance (in stained smears)*	*Sheath*	*Stylets*	*Body Nuclei (in tail)*
W. bancrofti	230–300	Smooth, sweeping coils	Present	1	Do not extend to tip
W. malayi	175–250	Irregular, kinky coils	Present	2	Extend to tip; last two swollen
L. loa	250–300	Irregular, kinky coils	Present	1	Extend to tip
M. ozzardi	185–200	Smooth curves	Absent	1	Do not extend to tip
A. perstans	190–200	Smooth curves	Absent	0	Extend to tip
O. volvulus	150–285 or 285–370	Smooth, sweeping coils	Absent	0	Do not extend to tip

swollen tip in *W. malayi,* is usually recurved in *L. loa,* is sharply pointed in *O. volvulus* and *M. ozzardi,* and bluntly pointed in *A. perstans.*

Anatomic Structures. In the column of body nuclei seen after staining, there are certain definite non-nucleated or clear areas which represent regions where various organs will be formed. These are the *nerve ring,* the *excretory pore* or "V" spot, and the *anal pore* or tail-spot.

The nucleus of the excretory cell and the four nuclei of the so-called genital cells are more prominent than others. Although these latter cells have usually been considered to be the primordia of the genital organs, it has been shown that they are more likely associated with the rectum and anus. A collection of granules found in the posterior half of the body of some microfilariae is called the "Binnen Körper" or inner body. These features are more or less constant in position in a given species and are usually expressed as percentages of the total body length, measuring the distance to them from the anterior end. Certain precautions should be understood before placing too much dependence upon these landmarks: (1) It is extremely difficult for even the expert to measure these distances accurately to a fraction. (2) Only faultlessly fixed and stained specimens are satisfactory for study.

Distance Between Anatomic Structures and Anterior Tip

	W. bancrofti	*W. malayi*	*L. loa*
Nerve ring	20%	26%	20%
"V" spot	29%	35%	30%
Tail-spot	83%	90%	84%
Excretory cell	31%	36%	36%
First genital cell	70%	70%	71%

Reaction to Stain. Anatomic features are more easily stained in some species than in others. Thus *W. malayi* is much more difficult to stain well than is *W. bancrofti,* and dilute Giemsa which brings out the sheath of the latter fails to stain this structure in *Loa loa.* The reaction of living microfilariae to methylene blue is sometimes used to separate species, especially *W. bancrofti* and *L. loa;* the former absorbs the stain while the latter does not.

Other Diagnostic Aids. Adult worms obtained by biopsy are almost always females and are difficult to identify (see key, Chapter 24). The cuticular markings and the structure of the anterior and posterior ends are the characters most used.

Considerable work has been done recently on skin and complement-fixation tests (Goodman et al., 1945; Zarrow and Rifkin, 1946; Wharton and Stelma, 1946; Warren et al., 1946; Saunders et al., 1946). The reaction is a group one, and antigen prepared from the closely related filaria, *Dirofilaria immitis,* of the dog may be used in the diagnosis of any one of these filarial diseases. This antigen has been used especially for Bancroftian infections in American service personnel in the early stage of the disease. Various workers have found the intradermal test to give approximately 90 per cent or more positive results with very few or no false positive reactions. Bozicevich et al. (1947) found that an antigen made from *O. volvulus* was fairly specific for onchocerciasis.

The laboratory diagnosis of infection with *Dracunculus medinensis* is best made by recovering larvae from the blister formed in the skin of the host. Immersion of the part in water will stimulate the discharge of the embryos. Serologic tests are available but are not of much value.

Roentgen-ray evidence of calcified adult filarial worms or the guinea worm in the subcutaneous tissues can be obtained in some cases. Recently Highby (1946) has used xenodiagnosis in cases of filariasis.

CHAPTER 24

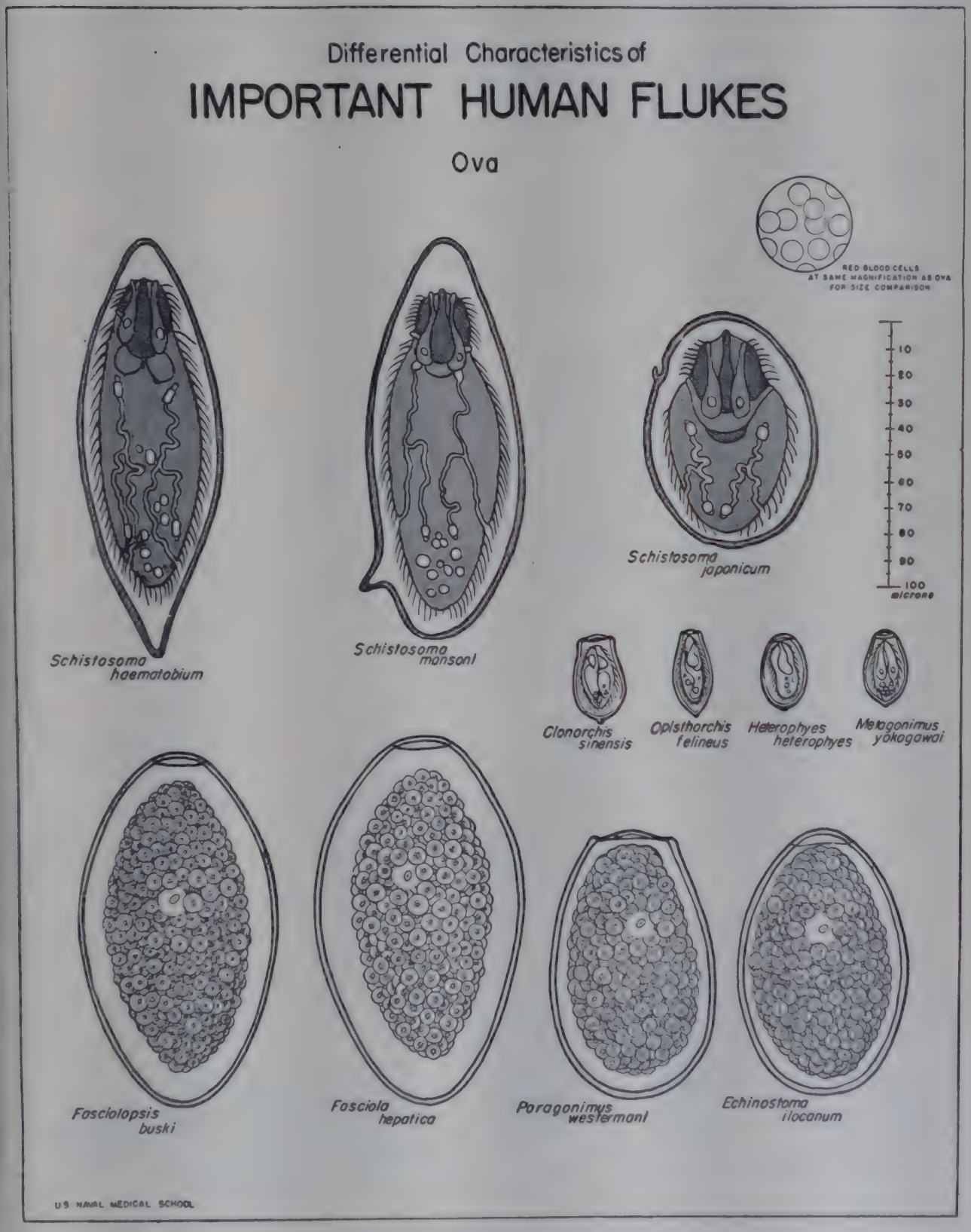
Keys to the Helminths and Laboratory Technics

The Acanthocephala, Gordiacea, and Hirudinea which include only rare, accidental, or spurious parasites of man are separated in the introductory key and described in Chapter 16; they are omitted in the following. The keys apply only to specimens recovered from man or his excreta.

Key to the Ova of Helminths

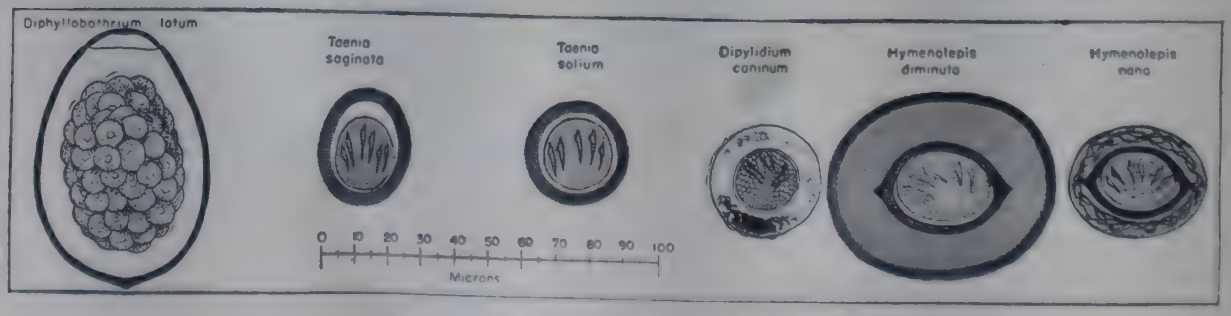
1. Ovum with an operculum, sometimes inconspicuous 2
   Ovum nonoperculate 11
2. Ovum small, less than 35μ, containing a developed larva when passed 3
   Ovum large, over 50μ, without developed larva when passed 6
3. Organs of larva, particularly the cytolytic glands, asymmetrical 4
   Organs of larva symmetrically arranged 5
4. Ovum with pronounced shoulders; bulb-shaped; size, 27 to 35 $\times$ 12 to 20μ; in feces or from duodenal drainage ..... *Clonorchis sinensis*
   Ovum with slight shoulders; not bulb-shaped, but more slender, tapering toward each pole; size, 30 $\times$ 12μ; in feces or from duodenal drainage ..... *Opisthorchis felineus*
5. Ovum widest below middle; size, 26 to 28 $\times$ 15 to 17μ; in feces ..... *Metagonimus yokogawai*
   Ovum widest at middle; size, 20 to 30 $\times$ 15 to 17μ; in feces ..... *Heterophyes heterophyes*
6. Ovum ovoid in shape 7
   Ovum rhomboidal, broadest in middle and tapering toward both poles; size, 160 to 170 $\times$ 60 to 70μ; in feces ..... *Gastrodiscoides hominis*
7. Ovum very large, over 130μ in length 8
   Ovum medium-sized, less than 120μ in length 9
8. Yolk granules evenly distributed throughout yolk cells; size, 130 to 140 $\times$ 80 to 90μ; in feces ..... *Fasciolopsis buski*
   Yolk granules concentrated around nuclei of yolk cells; size, 130 to 150 $\times$ 65 to 90μ; in feces or from duodenal drainage ..... *Fasciola hepatica*
9. Operculum very distinct and flattened with definite shoulders; dark golden-brown in color; size, 75 to 120 $\times$ 45 to 65μ; in sputum; in feces in one-third to one-half of the cases ..... *Paragonimus westermani*
   Operculum not flattened, often indistinct; yellowish-brown in color; in feces 10
10. Relatively thick-shelled; broadly barrel-shaped with broad operculum; size, 55 to 75 $\times$ 40 to 55μ ..... *Diphyllobothrium latum*
    Relatively thin-shelled; oval in shape with narrow operculum; size, 85 to 115 $\times$ 55 to 70μ ..... *Echinostoma ilocanum*
11. Ovum contains a ciliated larva; with conspicuous spine or minute knob 12
    Ovum does not contain a ciliated larva; without a spine or minute knob 14

12. Ovum with a conspicuous spine 13
    Ovum with a minute knob (frequently difficult to demonstrate); size, 70 to 105 × 55 to 80μ; in feces ..... *Schistosoma japonicum*
13. Ovum with terminal spine; size, 110 to 170 × 40 to 75μ; in urine, rarely in feces ..... *Schistosoma haematobium*
    Ovum with lateral spine; size, 110 to 180 × 45 to 75μ; in feces or biopsy of rectal ampullae, rarely in urine ..... *Schistosoma mansoni*



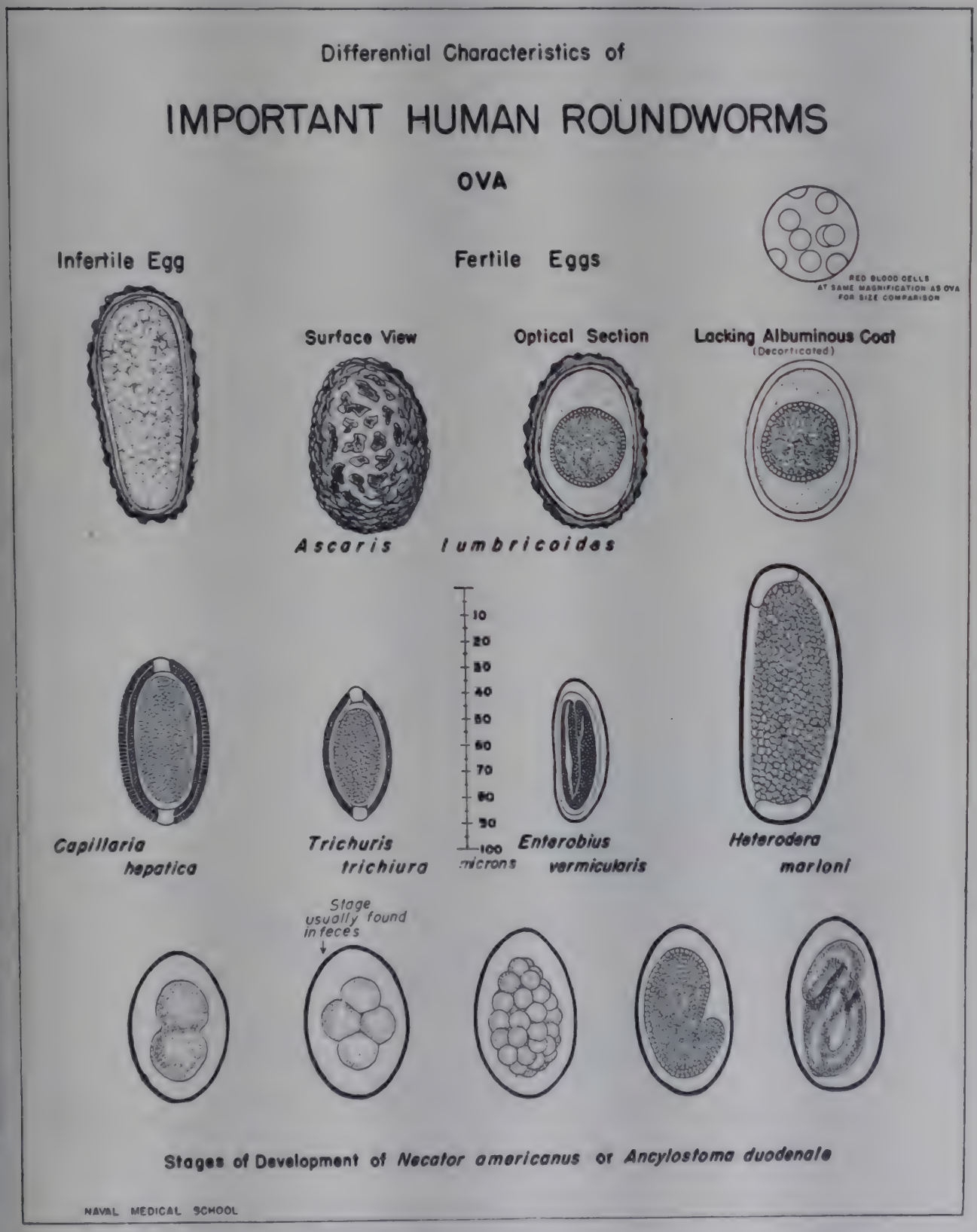
Ova of important human trematodes.

14. Ovum contains an embryo with three pairs of hooklets 15
    Ovum embryonated or unembryonated but never with hooklets 18
15. Ovum with a single thick, brown, radially pitted shell (embryophore); spherical (diameter, 30 to 50μ) or subspherical (30 to 40 $\times$ 20 to 30μ) ..... *Taenia* species (must be differentiated by adults)
    Ovum with outer and inner shell separated by space; outer shell moderately thin without radial pitting; inner shell thin, colorless 16
16. Ovum single; inner shell with polar thickenings 17
    Ova in packets of usually 10 to 12; inner shell without thickenings; outer shell transparent; spherical; diameter, 20 to 50μ ..... *Dipylidium caninum*
17. Outer shell transparent; inner shell with polar filaments; diameter, 30 to 60μ ..... *Hymenolepis nana*
    Outer shell light yellowish-brown; inner shell without polar filaments; size, 70 to 85 $\times$ 60 to 80μ ..... *Hymenolepis diminuta*
18. Ovum with dark, brown shell 19
    Ovum with clear, transparent shell 22
19. Ovum barrel-shaped; with mucoid plug at each pole; with smooth shell 20
    Ovum oval or ovoid, without mucoid plugs at the poles; with rough mammillated shell 21
20. Shell with radial striations; size, 50 to 65 $\times$ 30 to 35μ; rare, spurious parasite ..... *Capillaria hepatica*
    Shell without radial striations; size, 50 to 54 $\times$ 22 to 23μ ..... *Trichuris trichiura*
21. Ovum broadly oval; size, 45 to 75 $\times$ 35 to 50μ ..... *Ascaris lumbricoides* (fertile)
    Ovum elongate, oval, or rhomboidal; size, 88 to 95 $\times$ 40 to 45μ ..... *Ascaris lumbricoides* (infertile)
22. Shell very thick; rare 23
    Shell thin; much more common forms 24
23. Contents not segmented; size, 43 to 68 $\times$ 33 to 48μ ..... *Ascaris lumbricoides* (decorticated)
    Developed to larva stage; size, 50 $\times$ 35μ ..... *Physaloptera caucasica*
24. Ovum contains a developed rhabditoid larva 25
    Ovum contains embryos in various stages of development but not beyond morula stage 27
25. Ovum flattened on one side; size, 50 to 60 $\times$ 20 to 30μ ..... *Enterobius vermicularis*
    Ovum not flattened on one side, oval 26
26. Rare, only in diarrheal stools; size, 50 to 58 $\times$ 30 to 34μ ..... *Strongyloides stercoralis*
    Rare, only in old or constipated stools; size, 56 to 76 $\times$ 35 to 70μ; (must be differentiated by adults or filariform larvae) ..... Hookworm species
27. Ovum bean-shaped with air spaces at poles; developed to morula stage; size, 80 to 120 $\times$ 25 to 45μ; rare, spurious parasites .... *Heterodera marioni*
    Ovum oval 28



Ova of important human cestodes.

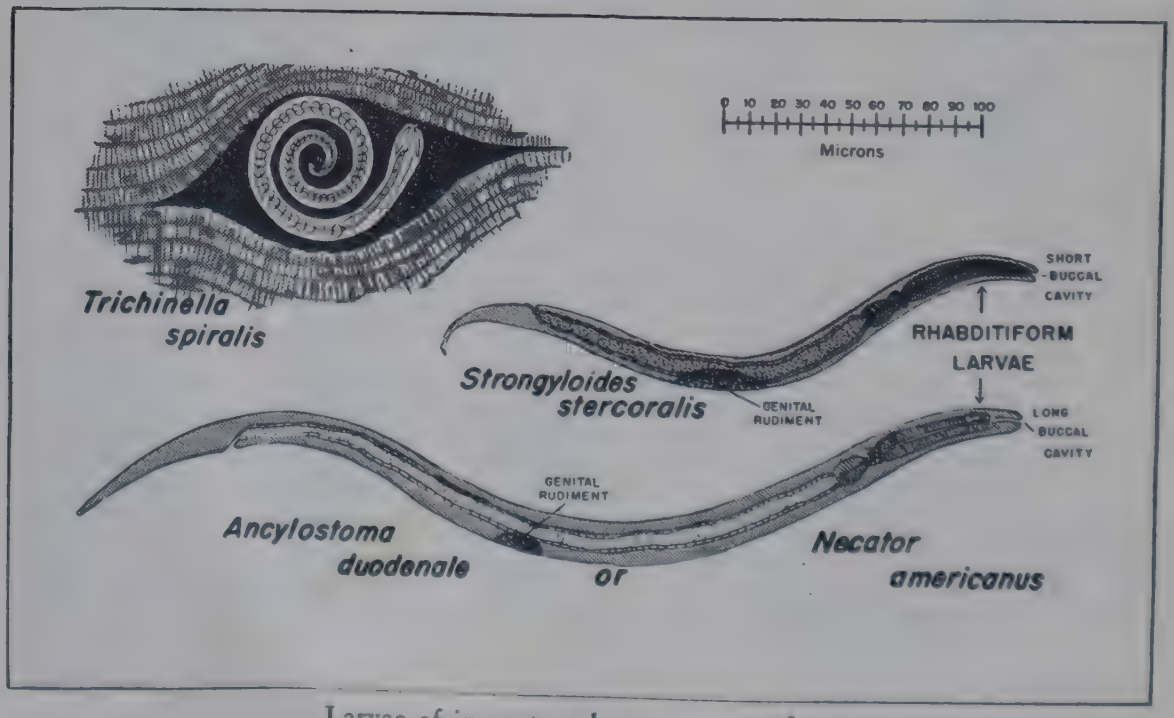
28. Both poles of egg broadly rounded 29
    One pole of egg more pointed than other; embryo usually developed to morula stage; size, 70 to 105 × 35 to 55μ ... *Trichostrongylus* species
29. Embryo usually developed to four-celled stage; size, 56 to 76 × 34 to 50μ; common ..... *Ancylostoma* species, *Necator americanus* (must be differentiated by adults or filariform larvae)
    Embryo usually developed beyond four-celled stage; size, 80 × 50μ; rare ..... *Ternidens deminutus*



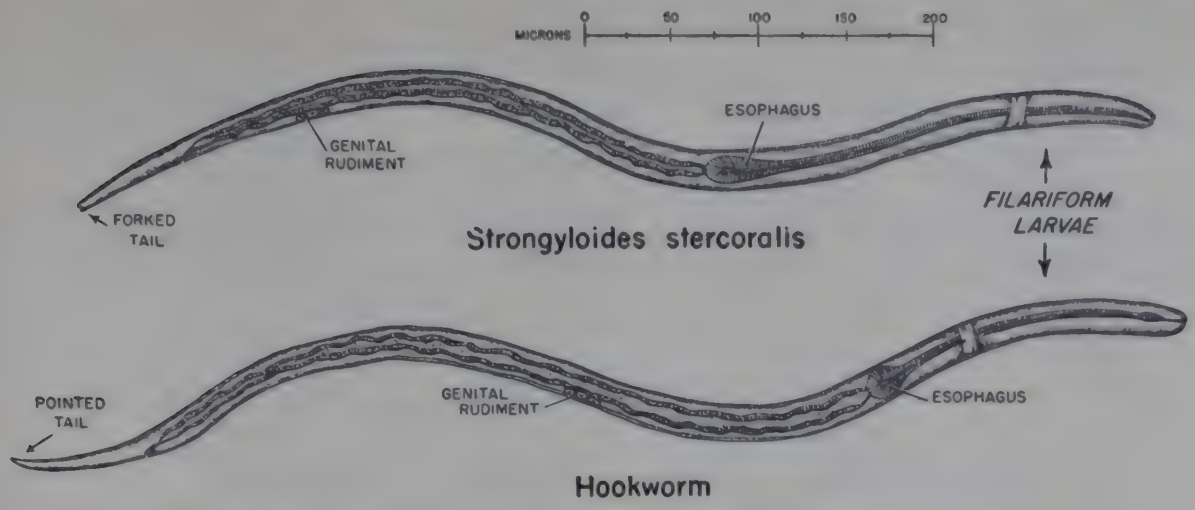
Ova of important human intestinal nematodes.

Key to the Larval Forms of Helminths

1. Larval form a nematode, a threadlike cylindrical worm most commonly recovered from feces, blood, or sputum, in only a few cases from tissues .....
   Larval form a cestode, a round, oval, or amorphous body usually with a typical tapeworm scolex, often showing hooks and suckers; almost always recovered by tissue biopsy, rarely from sputum, never from feces or blood 1
2. Larvae—forms in which some of the internal organs, usually the intestine, can be demonstrated .....
   Microfilariae—forms in which the internal organs are in an embryonic state of development, being represented by configurations of body nuclei 1
3. Larvae recovered from feces or by fecal culture .....
   Larvae recovered from other excreta or from tissue 1
4. Esophagus rhabditiform or pseudorhabditiform in type .....
   Esophagus filariform or pseudofilariform in type .....
5. Tail short, ends in a point .....
   Tail long, filamentous, usually ending in a minute knob .... *Trichostrongylus* specie
6. Esophagus with only a posterior muscular bulb; genital primordium large or small .....
   Esophagus with a median and posterior muscular bulb; genital primordium small, about 20μ in length; size, 0.24 to 0.3 mm. .... *Rhabditis* specie
7. Buccal cavity short, about equal to body width in length; genital primordium large, about 35μ in length; size, 0.2 to 0.25 mm. ..... *Strongyloides stercoralis*
   Buccal cavity longer than body is wide; genital primordium small, about 20μ in length; size, 0.25 to 0.3 mm. ..... *Ancylostoma* species, *Necator americanus*
   (must be differentiated by filariform larvae or adults)
8. Tail smooth .....
   Tail tuberculated ..... *Trichostrongylus* specie
9. Tip of tail ends in sharp point; genital primordium near middle of body 10
   Tip of tail minutely notched; genital primordium behind middle of body; size, 0.5 to 0.7 mm. ..... *Strongyloides stercoralis*



Larvae of important human nematodes.



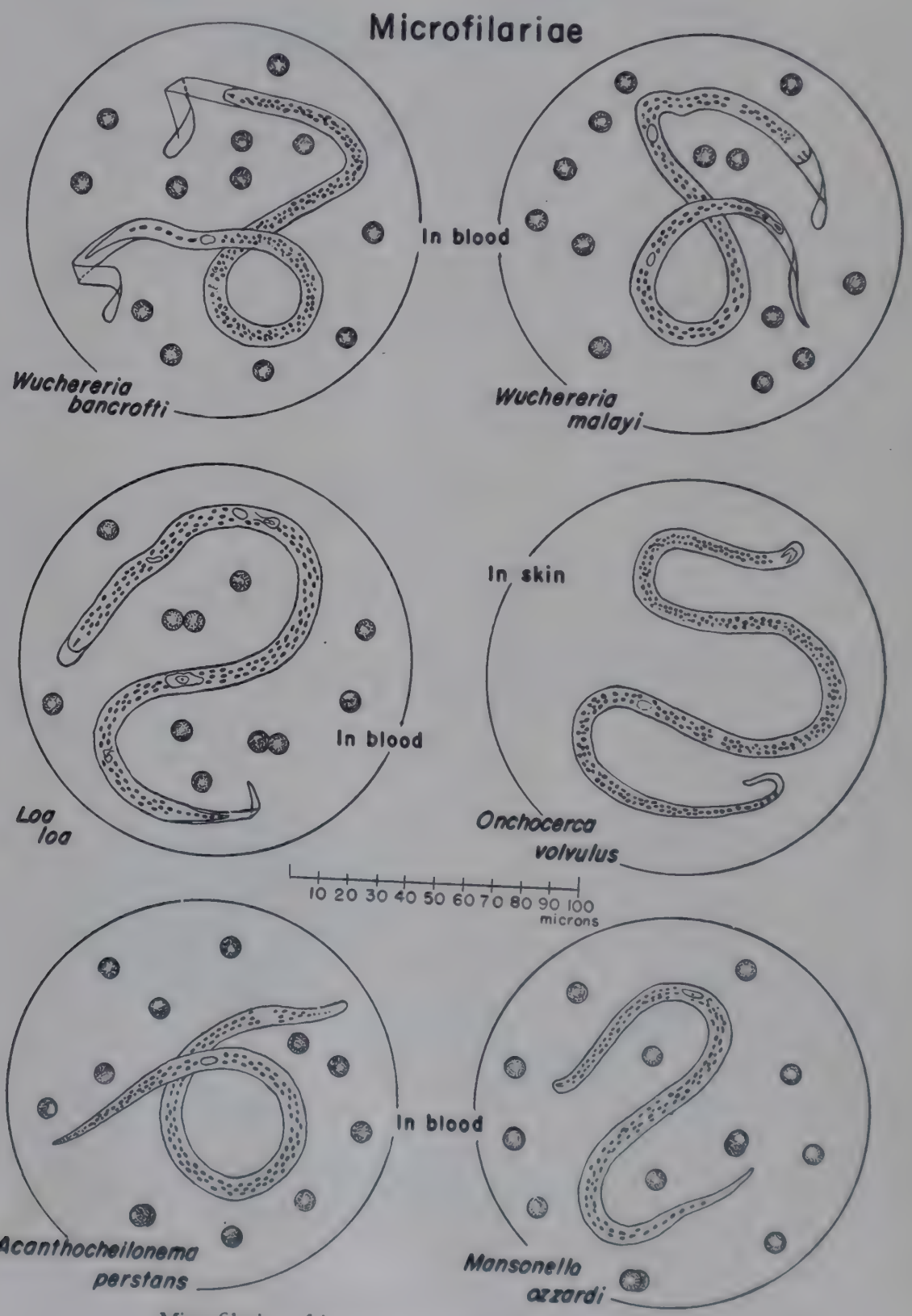
Filariform larvae of important human intestinal nematodes.

10. Head slightly flattened; without definite constriction at the esophageal-intestinal junction; esophageal spears unequal and inconspicuous; size, 0.6 to 0.8 mm. ..... *Ancylostoma duodenale*
    Head not flattened; with marked constriction at esophageal-intestinal junction; esophageal spears equal and conspicuous; size, 0.6 to 0.8 mm. ..... *Necator americanus*
11. Larvae recovered from sputum 12
    Larvae recovered from spinal fluid, blood, or tissue 13
12. Esophagus rhabditiform in type; size, 0.3 to 2 mm. ..... *Strongyloides stercoralis, Ascaris lumbricoides*
    (See couplet 7 for differentiation of species)
    Esophagus filariform in type; size, about 0.7 mm. ..... *Ancylostoma* species
    Necator americanus, Strongyloides stercoralis (rare)
    (See couplet 9 for differentiation of species)
13. Larvae recovered by muscle biopsy (size, 0.1 to 1 mm.) or rarely from blood or spinal fluid (size, about 0.1 mm.) ..... *Trichinella spiralis*
    Larvae recovered from fluid taken from skin blister; size, about 0.6 mm. ..... *Dracunculus medinensis*
14. Microfilariae in blood, rarely in fluid aspirated from a hydrocele or lymph gland 15
    Microfilariae in fluid aspirated from a skin nodule, or teased out from a skin snip; size, 150 to 370μ ..... *Onchocerca volvulus*
15. Microfilariae with sheath 16
    Microfilariae lacking sheath 18
16. Body nuclei do not extend to tip of tail; one stylet; lies in smooth sweeping curves; size, 230 to 300μ ..... *Wuchereria bancrofti*
    Body nuclei extend to tip of tail; lies in irregular kinky coils 17
17. Tip of tail swollen at region of last two nuclei; head not flattened; two stylets; size, 175 to 250μ ..... *Wuchereria malayi*
    Tip of tail tapers to point; head flattened, one stylet; size, 250 to 300μ *Loa loa*
18. Body nuclei extend to tip of tail; size, 190 to 200μ ..... *Acanthocheilonema perstans*
    Body nuclei do not extend to tip of tail; size, 185 to 200μ ..... *Mansonella ozzardi*
19. Larvae with hooklets and suckers; usually round or oval 20
    Larvae with anterior invagination without suckers or hooklets; usually elongated ribbon-like (plerocercoid or sparganum); size, about 3 to 30 mm. *Diphyllobothrium* species
20. Larvae with single bladder and scolex armed with hooklets (cysticercus); size, about 10 × 5 mm. ..... *Taenia solium*

Larvae with many bladders and many armed scolices (echinoccocus or hydatid); usually single scolices are recovered which are about 150μ long ..... *Echinococcus granulosus*

Key to the Adult Helminths

1. Without complete alimentary canal—mouth and intestine present or absent, always without anus; body more or less flattened; with hold-fast organs in the form of grooves or suckers 2

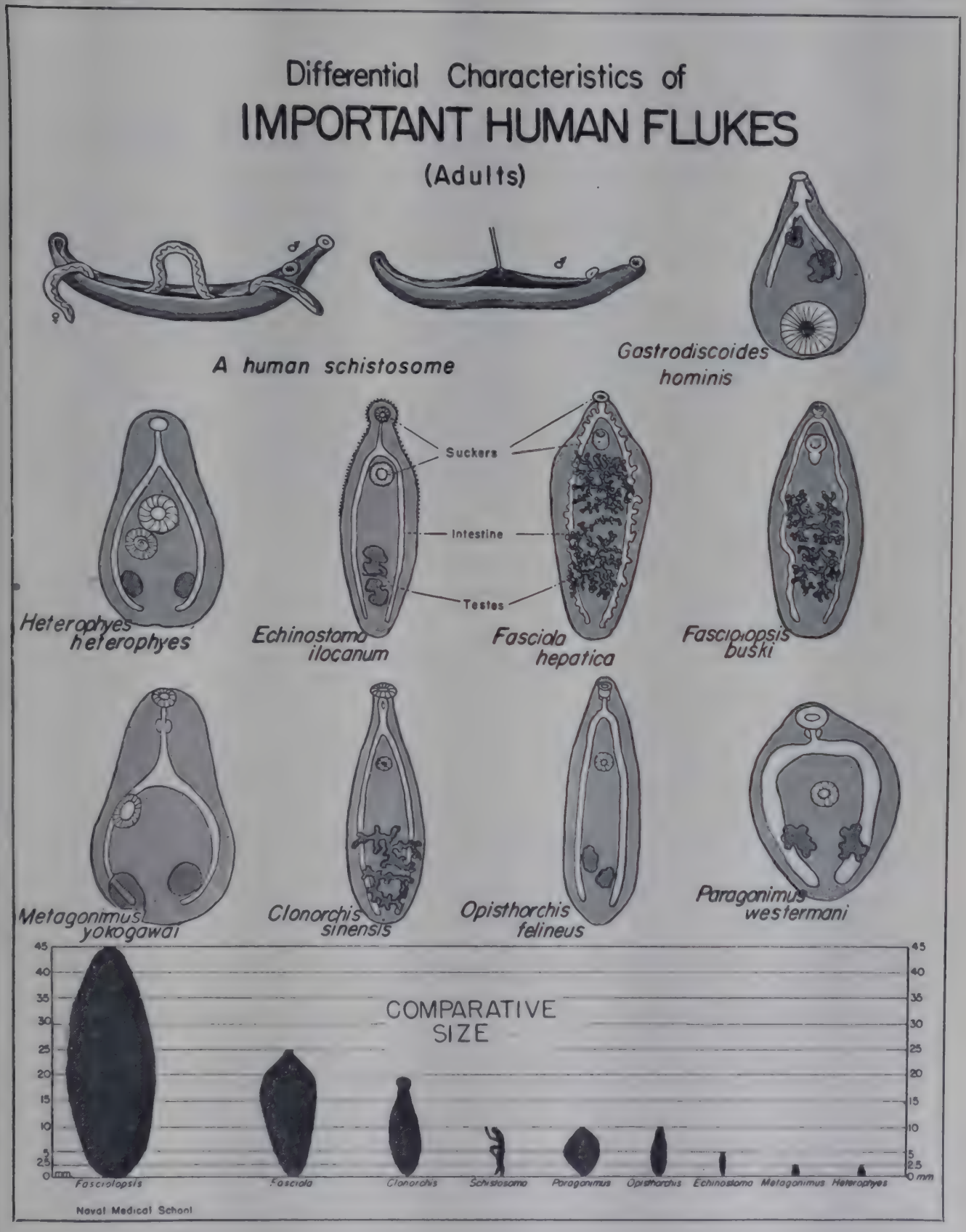


Microfilariae of important human somatic nematodes.

With complete alimentary canal—mouth, intestine, and anus present; body round in cross section; without suckers ..... Class Nematoda

2. Body of adult not segmented; one or two suckers present; mouth and intestine present; parasitic in liver, lungs, blood, intestine, occasionally elsewhere ..... Class Trematoda

Body of adult segmented; with two sucking grooves or four suckers; alimentary tract absent; adults parasitic in intestine ..... Class Cestoidea



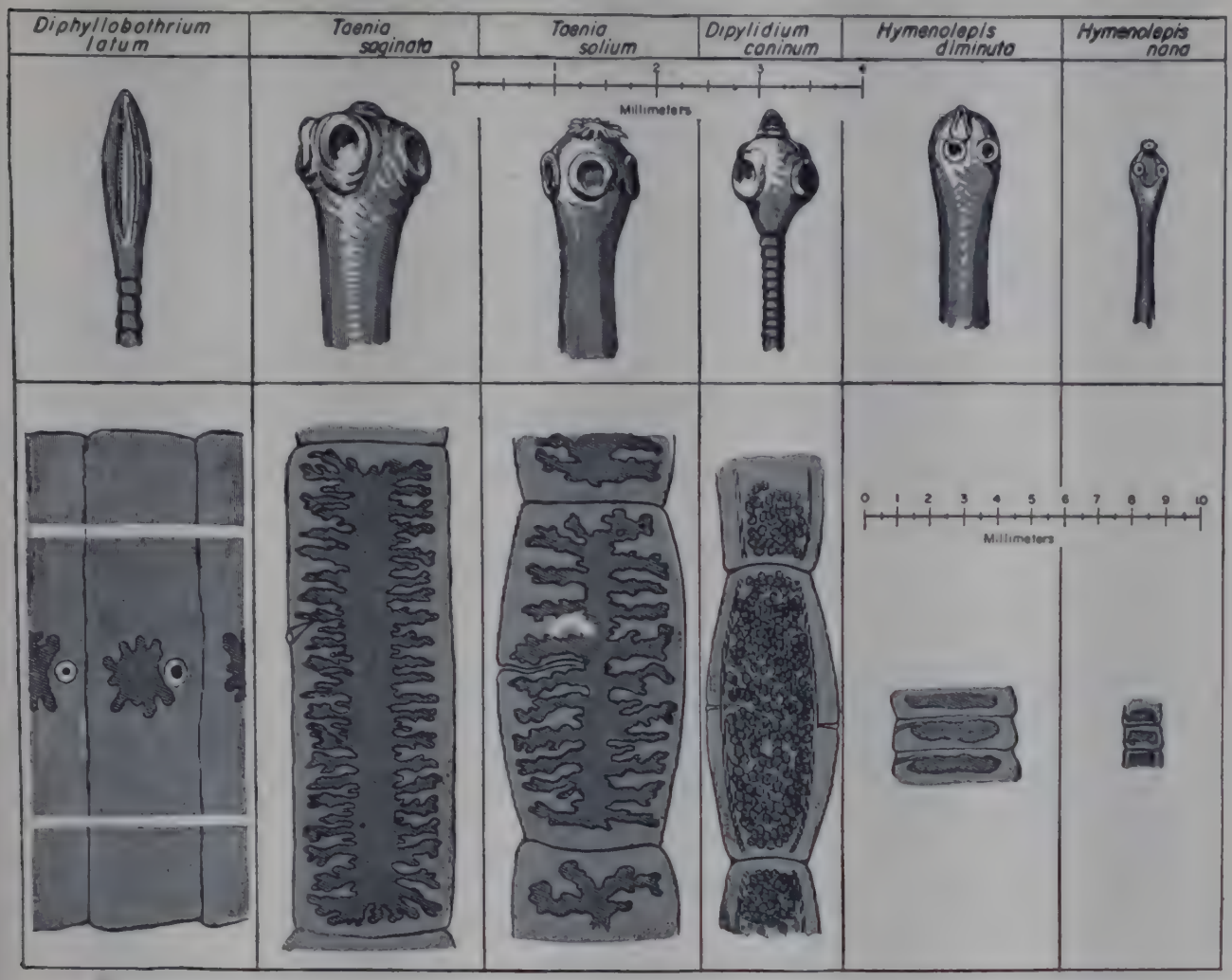
Adults of important human trematodes.

Class Trematoda

1. Sexes separate (dioecious) 2
   Sexes united (monoecious or hermaphroditic) 4
2. Male with finely tuberculated or smooth integument; female with large number of eggs in uterus 3
   Male with grossly tuberculated integument; female with not more than four eggs in uterus ..... *Schistosoma mansoni*
3. Male with four testes; egg from uterus of female showing terminal spine ..... *Schistosoma haematobium*
   Male with seven testes; egg from uterus of female with rudimentary lateral spine ..... *Schistosoma japonicum*
4. Ventral sucker usually anterior to middle of body, never at posterior end 5
   Ventral sucker conspicuous, at or near posterior end of body ..... *Gastrodiscoides hominis*
5. With only an oral and ventral sucker 6
   With a large genital sucker in addition to the oral and ventral suckers ..... *Heterophyes heterophyes*
6. With a circlet of prominent spines around the oral sucker ..... *Echinostoma ilocanum*
   Without such a circlet of spines 7
7. Intestinal ceca unbranched 8
   Intestinal ceca with secondary branchlets ..... *Fasciola hepatica*
8. Less than 2 cm. in length; testes lobed or nonlobed but never with fine branches 9
   Two to 7 cm. in length; testes extremely branched ..... *Fasciolopsis buski*
9. Testes lobed 10
   Testes not lobed ..... *Metagonimus yokogawai*
10. Testes with few primary lobes 11
    Testes with many secondary lobes ..... *Clonorchis sinensis*
11. Testes deeply lobed ..... *Paragonimus westermani*
    Testes weakly lobed ..... *Opisthorchis felineus*

Class Cestoidea

1. Scolex or "head" recovered 2
   Gravid proglottis recovered 7
2. Scolex with two longitudinal, slitlike grooves or suckers; about 1 mm. in diameter ..... *Diphyllobothrium latum*
   Scolex with four suckers 3
3. Scolex with rostellum armed with hooks 4
   Scolex unarmed 6
4. Scolex globular, large, about 1 mm. in diameter, armed with two rows of hooks ..... *Taenia solium*
   Scolex club-shaped, small, less than 0.5 mm. in diameter 5
5. Scolex armed with one row of hooks; about 0.3 mm. in diameter *Hymenolepis nana*
   Scolex armed with three to seven circlets of hooks; 0.25 to 0.5 mm. in diameter ..... *Dipylidium caninum*
6. Scolex pear-shaped, large, 1 to 2 mm. in diameter ..... *Taenia saginata*
   Scolex club-shaped, small, about 0.5 mm. in diameter ..... *Hymenolepis diminuta*
7. Segment longer than broad 10
   Segment broader than long 8
8. Genital pores ventral; uterus rosette-shaped ..... *Diphyllobothrium latum*
   Genital pores lateral; uterus a transverse elongated sac 9
9. Segments less than 1 mm. in breadth ..... *Hymenolepis nana*
   Segments about 2.5 mm. in breadth ..... *Hymenolepis diminuta*



Adults of important human cestodes.

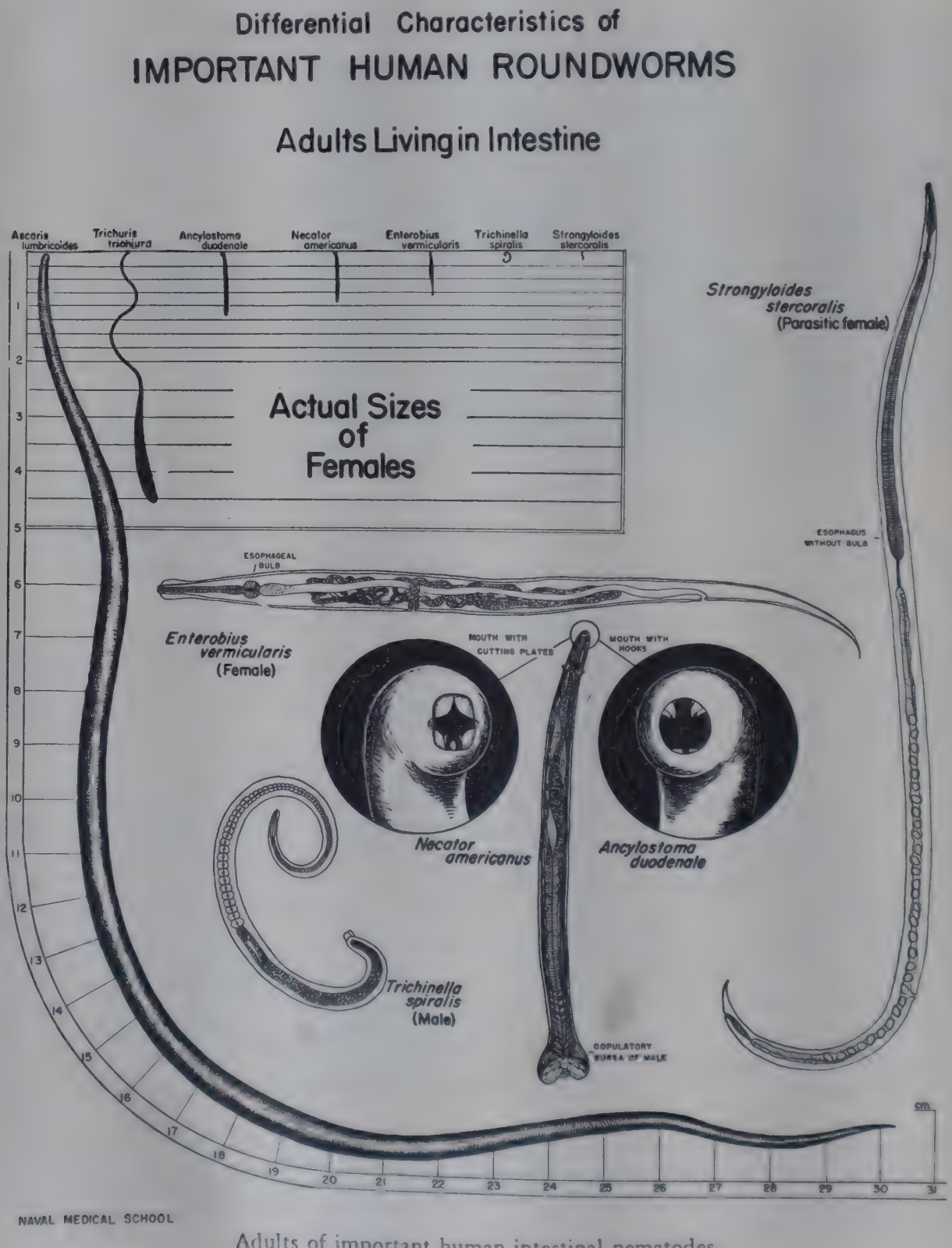
10. Uterus with median stem and a varying number of lateral branches; with single genital pore 11
    Uterus composed of a large number of pockets, each filled with ova; genital pores double ..... *Dipylidium caninum*
11. Uterus with 5 to 13 main lateral branches on each side ..... *Taenia solium*
    Uterus with 15 to 30 main lateral branches on each side ..... *Taenia saginata*

Class Nematoda

1. Worms with different regions of the body varying in diameter, usually tapering toward the extremities; usually found in the alimentary tract or embedded in the intestinal mucosa 2
   Worms long, slender and threadlike throughout entire length; found in tissues of man, never in the intestine 13
2. Worms with a more or less slender, threadlike anterior region and a thicker posterior portion; esophagus inconspicuous, with long posterior region composed of a small tube surrounded by a single row of glandular cells 3
   Worms tapering to either end but never as above; esophagus prominent, with heavily muscled posterior region and triradiate lumen 4
3. Large worms (female, 35 to 50 mm.; male, 30 to 45 mm.) with anterior end of body long and whiplike ..... *Trichuris trichiura*
   Small worms (female, 3 to 4 mm.; male, 1.4 to 1.6 mm.) with anterior end filiform but not whiplike ..... *Trichinella spiralis*
4. Anterior extremity markedly bent, males with a conspicuously widened copulatory bursa 5

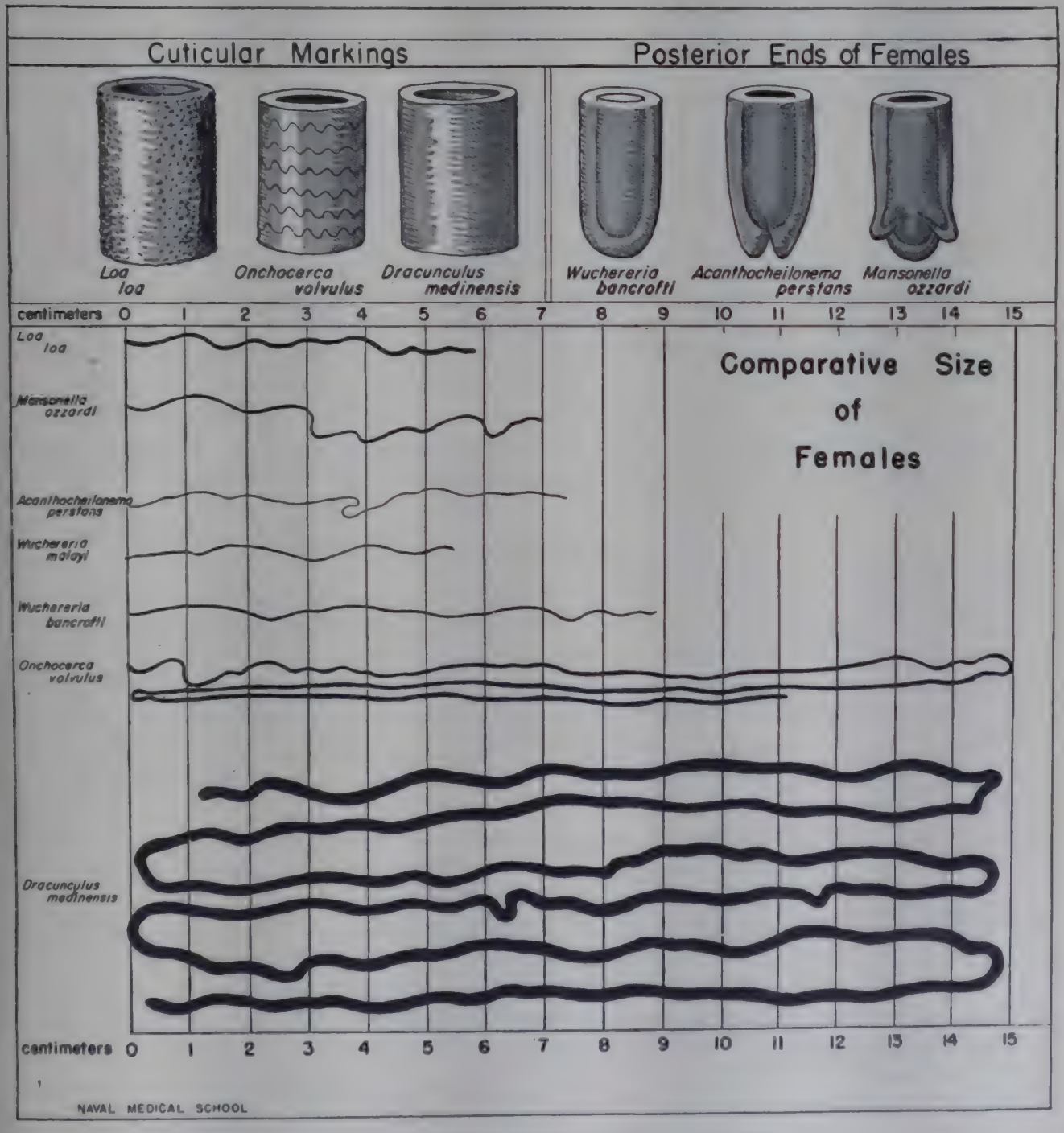
Anterior extremity straight or slightly curved, males with or without a copulatory bursa 8
..... 6

5. Mouth armed with hooklike teeth .....
   Mouth armed with cutting plates (female, 9 to 11 mm.; male, 5 to 9 mm.) ..... *Necator americanus*
   7
6. Two pairs of hooklike teeth .....
   Three well-developed pairs of hooklike teeth, the outermost the largest and the innermost the smallest (female, 14 mm.; male, 10 mm.) ..... *Ancylostoma caninum*



Adults of important human intestinal nematodes.

7. Outer pair of teeth large; smaller inner pair without accessory processes (female, 9 to 11 mm.; male, 8 to 8.5 mm.) ... *Ancylostoma braziliense*
   Teeth of equal size, the inner pair with a small accessory process (female, 10 to 13 mm.; male, 8 to 11 mm.) ... *Ancylostoma duodenale*
8. Small worms, less than 15 mm. in length 9
   Large worms, more than 100 mm. in length (female, 200 to 350 mm.; male, 150 to 310 mm.); male with curved posterior end ..... *Ascaris lumbricoides*
9. Anterior end of worm with conspicuous cervical alae; male without bursa copulatrix (female, 8 to 13 mm.; male, 2 to 5 mm.) ..... *Enterobius vermicularis*
   Anterior end of worm without cervical alae, male with or without bursa copulatrix 10



Adults of important human somatic nematodes.

10. Very small worms, less than 2.5 mm. in length; male without bursa copulatrix (female, 1.8 to 2.2.; male, 0.5 to 0.8 mm.) ..... *Strongyloides stercoralis*
    Medium-sized worms, more than 3.5 mm. in length; male with bursa copulatrix 11
11. Anterior end blunt; with mouth guarded by a circle of stout bristles (female, 12 to 16 mm.; male, 9.5 mm.) ..... *Ternidens deminutus*
    Anterior end tapering to point; without distinct buccal capsule 12
12. Spicules of male barbed (female, 5 to 7 mm.; male, 4 to 5 mm.) ..... *Trichostrongylus orientalis*
    Spicules of male pointed (female, 5 to 6 mm.; male, 4 to 5 mm.) ..... *Trichostrongylus colubriformis*
13. Cuticle with raised nodules or striations 14
    Cuticle smooth 15
14. Cuticle with nodules or minute bosses (female, 50 to 70 mm.; male, 30 to 35 mm.) ..... *Loa loa*
    Cuticle with annular striations (female, 115 to 700 mm.; male 20 to 40 mm.) ..... *Onchocerca volvulus*
15. Females very long (500 to 1200 mm.); males rarely found, deep in tissues (12 to 29 mm.) ..... *Dracunculus medinensis*
    Females less than 120 mm. in length; males rarely found 16
16. Tail of female bluntly rounded 17
    Tail of female with appendages 18
17. Tail of male ends in a sharp curve (female, 80 to 100 mm.; male, 40 mm.) ..... *Wuchereria bancrofti*
    Tail of male ends in about three spiral coils (female, 55 mm.; male, 22 to 23 mm.) ..... *Wuchereria malayi*
18. Tail of female bifurcated; head with cuticular shield (female, 70 to 80 mm.; male, 45 mm.) ..... *Acanthocheilonema perstans*
    Tail of female with two fleshy appendages; head unarmed (female, 65 to 80 mm.; size of male not known) ..... *Mansonella ozzardi*

Laboratory Methods for Helminths

The laboratory methods used to demonstrate the diagnostic stages of helminths are many and diverse. Few or none of them are without disadvantages and only a small number have general adaptability. The laboratory technician must weigh many factors before deciding which technics to use. He must answer such questions as: What are the most common and important helminths in this area? How many specimens will there be per day? How much apparatus is available? How many technicians will be working? Is it important to discover all infections or only the heavier ones? Are quantitative results desired?

Only those procedures which are believed to have practical application have been included here. An attempt has been made to select methods suited to the most primitive conditions as well as to the better-equipped laboratories.

Examination of Feces. Feces should be examined for the ova, larvae, or adults of all the flukes (other excreta preferable for *S. haematobium* and *P. westermani*), the intestinal tapeworms, and the intestinal roundworms (other methods preferable for *Enterobius*). In most instances several methods are available and at least the direct smear and one of the more refined technics should be employed before a negative report is rendered.

Stools should be collected in clean, dry containers. Examination should be made as soon as practical after collection, but in most cases helminthic material will remain identifiable for several days if the stool is kept in a cool environment. Specimens obtained

by purging or enemas may be positive when the ordinary stool is negative. Representative fecal portions may be placed in air-tight containers (metal pillboxes), packed and shipped to diagnostic laboratories.

The ova and larvae of the helminths may be found most efficiently under the low-power (16-mm.) objective. Most workers prefer a 10× ocular and a rather subdued illumination. After the parasites are found they may be more closely examined by the high-dry (4-mm.) objective; the oil-immersion objective is almost never required for identification. All preparations should be examined systematically beginning at one corner and traversing the preparation back and forth until all fields have been viewed (see diagram, p. 552).

DIRECT SMEAR. This is used as the first and all-purpose method for any stage of helminth which passes in the feces. At least three smears should be examined from three different portions of the specimen; as a rule the outside of the stool gives the highest percentage of positive findings. The ova of the blood flukes occur most frequently in small clots of blood and mucus. The technic is simple and a very small amount of apparatus is required. However, light infections are frequently missed and the method is not quantitative.

TECHNIC. A small specimen from the stool is thoroughly emulsified in saline or tap water by means of an applicator. Any large particles which would keep the coverglass from settling are removed. The smear should be heavier than that employed in searching for intestinal protozoa; newsprint should be just legible through it. A coverglass is placed on the preparation, air bubbles being avoided by the method described under intestinal protozoa (p. 552). The smear is examined systematically under the low-power lens of a compound microscope. Nematode larvae which are usually very active in the feces may be quieted and stained for identification by iodine, eosin, or other solutions.

STRAINING: 1. FOR OVA AND LARVAE. This method may be used for all helminth ova and larvae. The organisms remain in the filtrate from which the coarse roughage is removed. This method is of advantage in that much of the material which makes the parasites hard to find and recognize is removed. It is of greatest value when combined with other methods. It is of disadvantage in that there is little or no concentration of the parasites and in many cases ova are lost by the process. It is time-consuming and there is danger of contamination from a previously employed strainer.

Technic. Approximately 1 Gm. of feces is mixed in a small amount of water. This is passed through dampened cheesecloth or wire screen (20- to 120-mesh). Loopfuls of the filtrate are examined for parasites.

2. FOR ADULT HELMINTHS. This method is used particularly for recovering the smaller intestinal worms. Usually these parasites are only found subsequent to treatment; all stools should be examined for two or three days after the administration of the anthelminthic. This is the best method for recovering the smaller helminths and tapeworm scolices. It is time-consuming, however, and of little importance except to determine whether or not the scolex of a tapeworm has been eliminated.

Technic. The fecal specimen is mixed with enough normal saline to give the consistency of a thin paste. The mixture is strained through a graduated series of wire sieves (6-, 12-, 24-, and 40-mesh). The debris is rinsed and examined over a dark background by means of a hand lens or dissecting microscope using reflected light.

SEDIMENTATION. This method is used especially for the schistosome ova, the operculate eggs of the other flukes, and *Diphyllobothrium*. It may be used for all helminth ova which occur in the feces. Occasionally *Trichinella* adults may be found during the first few days of the infection by this method. The ova are not distorted; they are concentrated with a small amount of debris and may be easily preserved. However, the method is time-consuming and not quantitative. Effective concentration is not so high as that obtained by some of the other technics, especially those used for roundworm ova. Continuous flow sedimentation (Nelson and Bayliss, 1946) is a newer method which is promising but requires the test of time.

TECHNIC. The fecal specimen is thoroughly mixed in tap water until its consistency is

about that of a thin paste. The amount of water required usually falls between 10 and 20 times the volume of the fecal specimen. Mixing is facilitated if the first water added is slightly warm. The diluted specimen is then placed in a tall glass cylinder or a cone-shaped graduate and allowed to stand. After one to two hours the top three-fourths is siphoned or poured off, the cylinder refilled with water and the contents stirred well and allowed to stand for a second one- to two-hour period. The sedimentation process is repeated until the supernatant fluid is almost clear. (*Caution:* Schistosome ova may hatch if the procedure requires more than four hours.) Portions of the sediment are removed by means of a pipet to a glass slide, covered, and examined systematically with the low-power objective. (*Note:* Some workers prefer removing the coarse roughage before the first sedimentation by passing the fecal emulsion through a strainer (30- to 120-mesh). See straining methods for advantages and disadvantages.)

CENTRIFUGATION: 1. TAP-WATER METHOD. This is used for all helminth ova and larvae in feces. It is the best all-purpose concentration method. However, it is not so effective or simple as some other methods for specific helminths and is not quantitative. The method requires a centrifuge which may not be available.

Technic. A small amount of feces is mixed thoroughly in 10 to 20 times its volume of water until a thin paste is formed. This is strained (20- to 40-mesh) into a centrifuge tube with rounded bottom and spun one to two minutes at approximately 2500 revolutions. The supernatant solution is decanted; the tube is filled with water, its contents mixed thoroughly and centrifuged again. This process is repeated until the supernatant fluid is clear. (*Note:* For most laboratory diagnoses two spinnings are adequate.) The sediment is removed to a slide and examined.

2. ACID-ETHER METHOD. This is used especially for the schistosome ova. It gives about a five-fold increase over sedimentation methods in numbers of ova found. Its disadvantages are that it is expensive and lacks general applicability. The addition of a detergent, Triton NE, has given superior results in the hands of some investigators (Hunter et al., 1945). The modification of Loughlin and Stoll (1946) gives excellent results and may replace the older technics.

Technic. About 1 Gm. of feces is thoroughly emulsified in 5 ml. hydrochloric acid (40 ml. concentrated hydrochloric acid diluted to 100 ml.) in a test tube. The emulsion is filtered through two layers of moist gauze into a 15-ml. centrifuge tube. An equal quantity of ether is added; the tube is stoppered and shaken thoroughly. It is then centrifuged for one minute at 1500 revolutions. The debris at the acid-ether junction is loosened by ringing with an applicator stick. The acid and ether are poured off rapidly. The sediment is stirred in the remaining solution; a drop is decanted to the slide, covered, and examined systematically under the low-power objective.

FLOTATION. This method is used especially for the intestinal roundworm ova and for the eggs of certain intestinal tapeworms (*Hymenolepis nana* and *H. diminuta*). This is a very simple procedure and gives a highly effective concentration. It requires little apparatus and time. Its disadvantages are that it is not quantitative and is applicable only in regions where the flukes and tapeworms are not important. It misses infections of nearly all flukes, *Diphyllobothrium*, and a considerable percentage of *Taenia*, *Strongyloides*, and infertile *Ascaris*.

SOLUTIONS. Any one of the solutions listed below may be used. Sodium chloride (table salt) is the least expensive and very efficient if used correctly.

1. Table salt (crude). Saturated solution NaCl—sp. gr. about 1.20 or 1.21.
2. Table sugar. Saturated solution (2 lb. sugar, 1125 ml. water). As a preservative, 10 ml. phenol are added.
3. Calcium chloride. Saturated solution $CaCl_2$.
4. Zinc sulfate. 331 Gm. $ZnSO_4.7H_2O$ USP in 1 liter water. Sp. gr. 1.180. (A solution of sp. gr. 1.20 is used for formalin-preserved material.)

TECHNIC. About 1 Gm. of feces is thoroughly emulsified in a small amount of one of the above solutions in suitable container. Glass vials 2.5 cm. in diameter and 5.0 cm. tall are very satisfactory. The solution is added until it nearly fills the container. More is carefully added until a slight meniscus forms at the top of the vial. A slide (should cover entire surface area) is placed over the vial in contact with the meniscus. The covered vial is allowed to stand for 10 minutes to 1 hour (never over 30 minutes for brine). The slide is removed by lifting it straight up; it is inverted and examined systematically by the low-power objective. (*Note:* Alternative methods employ a coverglass instead of the glass slide over the meniscus or dispense with both slide and coverglass, a surface film being removed for examination by means of a loop.) For other recent ingenious methods see Egan (1941) and Eigenfeld and Schleisinger (1944). If desired the specimen may be strained before the ova are floated. This gives a clearer field but a certain percentage of the eggs are lost; it is usually not considered necessary.

COMBINED CONCENTRATION OF BOTH HELMINTH OVA AND PROTOZOAN CYSTS: ZINC-SULFATE CENTRIFUGAL FLOTATION (FAUST ET AL., 1938, 1939). This is the best method for combined concentration of both protozoan cysts and helminth ova. (See p. 554 for the technic.) It is applicable only to ova that will float and consequently has all the disadvantages of the flotation methods. It is inferior to direct flotation for helminths alone and requires more time and apparatus. It is not quantitative. It is recommended for laboratories which desire to diagnose both protozoan and worm infections in one procedure but not for helminths alone.

ESTIMATION OF WORM BURDEN (OVA-COUNTING). These methods are not advised for routine laboratory or field work. Their value lies in research and in studies to determine the worm burden of various populations. Probably the most efficient technic to give quantitative results for hookworm, *Ascaris,* and *Trichuris* ova is the direct centrifugal flotation method of Lane (1923). Stoll (1923) and Caldwell and Caldwell (1926) have also designed efficient ova-counting methods that can be highly recommended. All of the methods require careful technic and special apparatus. The original papers and their modifications should be carefully studied before the methods are employed. The Baermann technic can also be adapted for ova counting.

HATCHING TECHNIC. This is used especially for *Schistosoma mansoni* and *S. japonicum* ova (see examination of urine for the application of this method to *S. haematobium*). The method has a high efficiency in disclosing very light infections. It is a simple procedure requiring but little apparatus. However, it is time-consuming, and infertile and degenerate ova are missed.

TECHNIC. The fecal specimen is sedimented. This sediment is diluted with clear water and allowed to stand for 12 to 24 hours. The top few centimeters of water are examined with hand lens for free swimming miracidia. (*Caution:* Free swimming protozoans (ciliates) occur in stale water and may be confused with the trematode larvae.)

CULTURE METHOD. This is used primarily for hookworm and *Strongyloides* larvae. It is a simple technic which requires little apparatus. It has the disadvantage of being time-consuming.

TECHNIC. The fecal specimen is mixed with an equal quantity of powdered animal charcoal or sterile sand. Enough water is added to give a soft consistency. The mixture is placed on a piece of wet filter paper in a Petri dish and covered. The water of condensation on the underside of the cover is examined at various intervals or the larvae are recovered by using the Baermann technic.

PREPARATION OF ADULT HELMINTHS FOR IDENTIFICATION. Almost all adult worms can be identified macroscopically, or microscopically under a dissecting microscope or the low-power objective of a compound microscope. Gravid proglottides of tapeworms and adult flukes may be pressed between two slides and examined against a strong light or with low magnification; immersion in 75 parts carbolic acid and 25 parts xylene solution will clear the animals and is of value particularly for fixed specimens.

PRESERVATION OF FECAL SPECIMENS. This procedure is used in cases where immediate examination of the stool is not practical. The fecal specimen is diluted with tap water until a thin paste is formed. This mixture and an equal amount of steaming 10 per cent formalin are poured together into another vessel. This is set aside for a few hours. Then the supernatant solution is decanted and the formalin replaced. (*Note:* Formalin-preserved ova are heavier than those from unfixed stools. If flotation methods are used to concentrate them, solutions of 1.20 or higher must be employed.) Maplestone and Mukerji (1943) recommend adding 1 per cent sodium chloride in the proportions of 30 : 1 to the stool. Such fecal specimens may be sent to a laboratory; the ova will remain recognizable for about one month.

PRESERVATION OF HELMINTHS RECOVERED FROM FECES: OVA AND LARVAE. The immature stages of the helminths after concentration by sedimentation or centrifugation may be preserved in steaming 10 per cent formalin. If it is desired to keep the ova for periods longer than about one year they may be transferred gradually through a graded series of alcohols, 30 to 50 to 70 per cent.

ADULTS. A simple and practical method of fixation which is satisfactory for identification and preservation of laboratory specimens is given below. In cases where fine histologic detail is desired, more complex fixing solutions are required, and a text on microscopic methods should be consulted. The fixative used and technic employed will vary depending upon the species involved.

Technic. Living worms are placed in warm normal saline solution and shaken until the animals are completely fatigued. Steaming 5 per cent formalin is then poured over the relaxed worms while they are being shaken constantly to prevent contraction. Dead worms need only be washed with warm saline and dropped into steaming 5 per cent formalin. For the roundworms 10 ml. glycerin to 100 ml. of 5 per cent formalin, added before heating, may be recommended.

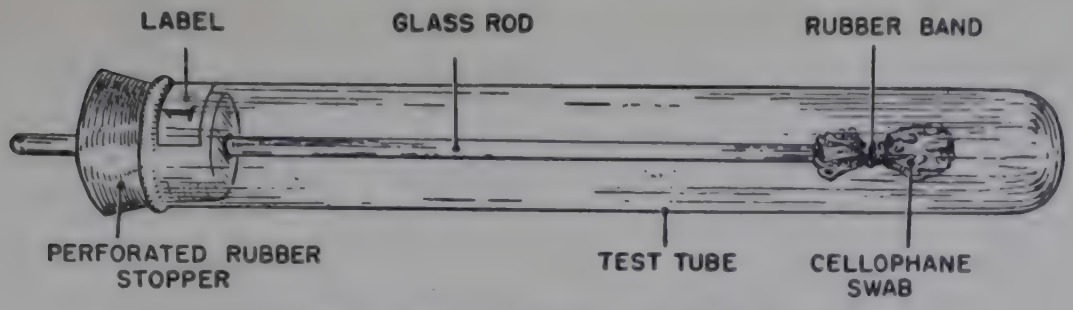
Examination of Perianal, Perineal, and Other Body Regions: PERIANAL AND PERINEAL AREAS. Removal and study of material from around the anus is the method of choice for the diagnosis of *Enterobius vermicularis.* Only 5 to 10 per cent of those infected with this parasite show ova in the feces. Some workers have recently reported higher positive findings for *Taenia saginata, Schistosoma mansoni,* and *S. japonicum* by this technic than when the feces alone are examined. *Ascaris* and *Trichuris* ova also may be found by this method but fecal examination is more efficient for these species. For best results the specimen should be obtained before defecation or bathing in the morning. It is recommended, that several specimens be taken on different days before a negative report is given. The pestle method of Schüffner and Swellengrebel (1943) and the slide technic of Petersen and Fahey (1945) are recent developments which may prove better than the technics given below. Watson and MacKeith (1946) discuss and describe various methods including a tissue paper swab and a camel's-hair brush technic.

NIH ANAL SWAB. This is used primarily for *E. vermicularis,* but is also applicable to the other species listed above. The advantages are the very simple technic combined with high efficiency. The scraping can be done by the patients or their parents and the specimen sent to the laboratory. However, a certain amount of preparation is required which is unnecessary if the specimens can be taken by the technician and immediately examined.

Technic. The perianal and perineal folds are scraped firmly with the prepared anal swab. The rod is replaced in the tube until ready for examination. Then the rubber band is cut and the cellophane is spread on a slide with a few drops of sodium hydroxide or water. This is covered with another slide or a heavy coverglass and examined microscopically.

SCOTCH-TAPE METHOD. This is used for the same species as the NIH swab. It is a very quick and efficient method requiring very simple apparatus. It is not so well adapted to use by the patient with later diagnosis in the laboratory as is the NIH swab.

Technic. A small strip of transparent scotch tape is placed, with the adhesive side out, over the rounded end of a small test tube. The test tube is rocked backward and forward



National Institute of Health (NIH) anal swab for pinworm.

on the perianal folds, allowing the tape to pick up the fecal debris. The tape is then placed, with sticky side down, on a slide and examined microscopically.

Fingernails. The fingernails are examined mainly for *Enterobius vermicularis* but they may harbor other species in which infection passes from the contaminated fingers to the mouth. Positive findings demonstrate one method of infection but do not indicate necessarily the presence of infection itself.

technic. The dirt is removed from under the nails with cotton soaked in 5 per cent sodium hydroxide. The cotton is then rinsed in a small amount of sodium hydroxide, and the solution is centrifuged. The sediment is examined microscopically. Some workers (Schüffner and Swellengrebel, 1944) prefer materials other than cotton in the above technic.

Nose. Essentially the same method is used in examination of the nose as that employed for the fingernails. For the nose, however, the examination is less reliable from a diagnostic standpoint. The nose is swabbed with dry cotton from which the sediment is removed and examined as is described above.

Examination of Sputum. Sputum is primarily examined for the ova of *Paragonimus westermani,* the lung fluke. In a very few cases, however, larvae and adults of *Strongyloides* and more rarely larvae of *Ascaris* and the hookworms have been found. In cases where a hydatid cyst of the lung has suppurated or a liver cyst ruptured into the lung, scolices and fragments of the cyst wall may be found in the sputum. Schistosome ova may also occur in the sputum (Silveira, 1944).

Direct Smear. This is used for all the species listed above, particularly the lung fluke. It is a simple method requiring little apparatus. It does not concentrate the parasites.

technic. The mouth is rinsed out thoroughly with hydrogen peroxide and the patient is asked to cough up sputum from lower respiratory passages into a cup. The sputum is examined microscopically as for fecal specimens, particular attention being paid to iron-brown specks which may be the ova of the lung fluke. In cases where echinococcosis is suspected, Best's carmine is recommended to differentiate the smaller fragments.

Centrifugation. This method is used especially for detection of lung-fluke ova. It gives an effective concentration of parasites. It requires more apparatus and time than does the simple smear.

technic. If the sputum is at all thick an equal amount of 3 per cent sodium hydroxide solution is added to it before centrifuging at high speed. After centrifugation the supernatant solution is decanted and the sediment examined microscopically.

Preservation of Lung-fluke Ova. Ova of the lung fluke may be preserved in a solution of 5 parts glycerin, 1 part phenol, and 94 parts water.

Examination of Urine. Urine is primarily examined for the ova of *Schistosoma haematobium;* microfilariae of *Wuchereria bancrofti* and larvae of *Strongyloides stercoralis* have also been recovered. Eggs of the dog kidney worm (*Dioctophyme renale*), a very rare parasite of man, are passed in urine.

Sedimentation. This method is used for the detection of ova of *Schistosoma haematobium.* The technic is the same as for examination of the feces except that no dilution is necessary.

Centrifugation. Centrifugation is useful for the detection of *S. haematobium* ova, *W. bancrofti* microfilariae, and *S. stercoralis* larvae. This technic is also the same as that for feces. No dilution or staining is necessary, however.

Hatching Technic. This is used for *S. haematobium*. The method is the same as for feces. *S. haematobium* miracidia are evenly distributed in the water, not concentrated at the surface.

Examination of Blood. Blood is examined for the microfilariae of all filarial worms except *Onchocerca volvulus*. The specimens should be collected at the most advantageous time of the day or night depending upon the species (see laboratory diagnosis of Filaria). Larvae of *Trichinella spiralis* occur in the blood from the sixth day after infection until the end of their migration period but can be demonstrated only in very heavy infections.

Fresh Smear. The fresh smear is examined primarily for microfilariae, which are easily seen under the low-power objective of the microscope. Microfilariae have a characteristic writhing movement. The method is a simple one requiring little apparatus; it misses light infections and is often not convenient.

Thin Blood Smear. For the detection of parasites the thin blood smear is made and stained like the thin smear for malaria (p. 506). Frequently light infections are missed and the stain is often not adequate for species differentiation.

Thick Blood Smear. This is made and stained with Giemsa like the thick smear for malaria. By using a measured quantity of blood, usually 20 cu. mm., it can be made roughly quantitative. If Wright's stain is used the smear must be dehemoglobinized. This is a very advantageous technic since both malaria and filariasis can be discovered on a single slide and by using a single stain. It is usually the method of choice.

Concentration: 1. centrifugation or sedimentation. Centrifugation or sedimentation is used for the detection of microfilariae and the larvae of *Trichinella spiralis*. Laked blood is usually used for the latter, in which the method is of value only in heavy infections. It affords a concentration of microfilariae and larvae. However, it is time-consuming, requires appropriate apparatus, and is seldom necessary since the thick smear will give nearly as good results.

Technic. Five to 10 ml. blood are obtained and citrated (10 ml. blood to 2 ml. 2 per cent sodium citrate in normal saline) or laked (10 ml. blood to 50 to 100 ml. 2 per cent acetic acid or 10/N hydrochloric acid). The citrated or laked blood is centrifuged at 1000 revolutions for 5 to 10 minutes. The sediment is then removed from the bottom of the tube and examined microscopically or a smear is made which is dried and stained. (*Note:* Sedimentation for 12 to 24 hours can be substituted for centrifugation if the blood is laked.)

2. knott concentration. This is used for filarial survey work, especially in areas where the microfilariae show nocturnal periodicity and it is more convenient to take day smears. The method is excellent for survey work and can be made quantitative. Harris and Summers (1945) have described a new and promising technic for the concentration of microfilariae.

Technic. One ml. blood is mixed with 10 ml. 2 per cent formalin in a centrifuge tube. The mixture is centrifuged at 1000 revolutions for five minutes or sedimented for 16 to 24 hours. The supernatant fluid is decanted, care being taken to get rid of surface bubbles. The sediment is aspirated with a capillary pipet and spread on a slide over areas about 22 mm. square. It is dried in air and fixed for 10 minutes in equal parts of ether and 95 per cent alcohol. The slide is dried in air and stained for 40 to 60 minutes with Delafield's hematoxylin. It is rinsed quickly in 0.05 per cent hydrochloric acid, washed in running water until the blue color appears, and dried in air. A drop of immersion oil is placed on the smear and examination is made systematically with the oil-immersion objective. Some workers prefer to place a coverslip on the preparation.

Special Staining Methods. Although the stains recommended above are usually adequate for finding the microfilariae they are often inadequate in bringing out the fine

details necessary for identification. The following methods are recommended in these instances and also when good permanent slides are desired.

VITAL STAINS. These are used to differentiate microfilariae of the various species. The method is simple requiring little apparatus; however, the slides are not permanent and it is often inconvenient to carry out this method of staining.

Technic. A very small drop of blood is mounted under a coverslip. A drop of 1 part methylene blue to 5000 parts physiological saline are drawn under the coverslip and the slip is ringed with petroleum jelly. If microfilariae are rare they may be concentrated in several cubic centimeters of blood by dehemoglobinization and centrifugation before staining.

HEMATOXYLIN STAINS. These are used when fine detail and permanent slides are desired. Bohmer's, Heidenhain's, Delafield's, or other hematoxylin stains may be used. The following method has given excellent results at the Naval Medical School.

Technic. A thick blood smear is made and thoroughly dried. The smear is dehemoglobinized for at least one hour during which the water is changed several times. After drying the slide is passed through or rinsed in the following solutions: methyl or absolute alcohol, 95, 80, 70 per cent, and tap water (3 min. each); Harris' hematoxylin (4 to 8 min.); rinsed in tap water; alcohol 50 per cent (3 min.); acid alcohol until pink; rinsed in 70 per cent alcohol and placed in alkaline alcohol until blue; eosin alcohol (2 min.); rinsed in 95 per cent alcohol and passed into absolute alcohol (3 min.); acetone, xylol, second xylol (5 min. each). Canada balsam is dropped on the blood film and a coverslip applied before all of the xylol has evaporated, otherwise the field will be milky and ruined. (*Note:* The time in the hematoxylin will vary between four and eight minutes. Both stain and counterstain timing may have to be adjusted. Particular attention should be given to decolorization.) Giemsa-stained specimens may be decolorized with acid alcohol and restained as above.

Solutions. Acid alcohol—2 ml. hydrochloric acid per 100 ml. 70 per cent alcohol by volume. Alkaline alcohol—sodium bicarbonate added to 70 per cent alcohol until the solution turns litmus paper blue. Eosin alcohol—4 per cent solution of eosin in 95 per cent alcohol.

Examination of Fluid Aspirated from a Hydrocele, Lymph Node, Skin Nodule, or Cyst. Microfilariae of *Wuchereria bancrofti* and *W. malayi* may be recovered from fluid taken from a hydrocele or enlarged lymph node; those of *Onchocerca* can be aspirated from skin nodules. Scolices are demonstrated from a hydatid cyst. The material removed may be studied in fresh smears or centrifuged and the sediment examined. For identification of species of microfilariae, smears are made and stained by dilute Giemsa or hematoxylin.

Examination of Fluid Removed by Duodenal Drainage. *Strongyloides stercoralis* larvae and the ova of the liver flukes may be recovered by duodenal drainage. Some workers report increased numbers of positive findings as compared to fecal examinations. However, duodenal drainage is not recommended for the diagnosis of parasitic infection except in cases where all other technics have been tried and there still remains a definite suspicion that one of the above species is involved.

TECHNIC. Sediment is obtained by centrifugation or sedimentation, and examined microscopically.

Examination of Material Obtained at Biopsy (or Autopsy). The presence of schistosomes, somatic tapeworms, and somatic roundworms which have a more or less prolonged phase in the tissues of man may be diagnosed by examination of biopsy material. Specimens are usually taken from the skin, subcutaneous tissues, lymph nodes, rectum or somatic muscles. The technics given below are also applicable to autopsy material.

COMPRESSION METHOD. This is used for *Trichinella spiralis* larvae encysted in muscle tissue. Small strips of deltoid, biceps, or gastrocnemius muscle are examined. It is the

method of choice for biopsy specimens. It should be combined with digestion for autopsy material. The technic is easy to perform and has a high degree of accuracy. However, it is not adapted to the handling of large amounts of tissue.

TECHNIC. One-gram sections are teased out on a compression slide and examined systematically under a dissection microscope or the low-power objective of a compound microscope. (*Caution:* Care must be exercised not to be misled by old infections.)

DIGESTION METHODS: 1. FOR TRICHINELLA SPIRALIS. In the detection of *Trichinella spiralis* the digestion method is used primarily for autopsy specimens, in which large amounts of tissue may be examined. It may be used for biopsy material also, but is not recommended since the compression method is quicker and more accurate for small amounts of tissue. The technic will not detect larvae less than 21 days old; calcified larvae are frequently missed.

Technic. Up to 200 Gm. of ground-up diaphragm or other muscle tissue are digested in a liter of digestive fluid (pepsin, 5 Gm.; hydrochloric acid, 155 ml.; water, q.s. to 1000 ml.). This is placed in an incubator at 37° C. for 12 to 18 hours; it is stirred occasionally and allowed to sediment. The supernatant fluid is discarded, and the remainder is screened (20- to 40-mesh) and resedimented twice in water of the same temperature. The sediment is examined under a dissecting microscope.

2. FOR SCHISTOSOMA MANSONI. The digestion method is used for both biopsy and autopsy material. Small bits of the rectal ampullae were biopsied by Ottolina and Atencio (1943) with a considerable increase in positive findings. Portions of the intestine, liver, and lung may be examined at autopsy. The compression method (see above) is also applicable and is recommended by some workers (Morales and Maldonado, 1945).

Technic. Small pieces of tissue are digested in 4 per cent potassium hydroxide at 60° to 80° C. for three hours. After sedimentation or centrifugation the sediment is examined with the low-power objective of the compound microscope.

PARASITES TEASED OUT IN NORMAL SALINE. This method is used for the cysticercus larvae of *Taenia solium* removed from the skin, subcutaneous tissues, or superficial musculature; the skin nodules containing *Onchocerca volvulus* adults and microfilariae; and the adults of *Wuchereria* species within biopsied lymph nodes.

FIXATION, SECTIONING AND STAINING OF HELMINTHS IN TISSUE. Portions of the biopsied tissue suspected of containing helminths, especially the somatic forms, should be fixed in Zenker's fluid (potassium dichromate, 2.5 Gm.; mercuric chloride, 5 to 8 Gm.; distilled water, 100 ml.; add 5 ml. glacial acetic acid just before use) and sectioned and stained by the usual pathologic methods.

Examination of Soil, Furniture, Etc.: RECOVERY OF LARVAE: BAERMANN METHOD. The Baermann method is used especially to recover hookworm and *Strongyloides* larvae from feces or after culture. It gives excellent results with a high effective concentration. It may also be used after culture to estimate the number of hookworm ova in a given specimen. It is time-consuming.

Technic. A short rubber tube is attached to the stem of a large glass funnel (preferably ribbed) and the funnel placed in a ring stand or other support. The rubber tube is shut off by clamp or other method. A specimen of soil, feces, or culture material is placed in a sieve of bronze or brass screening (1-mm. mesh) which is lined with cheesecloth and made to fit in the funnel. Lukewarm water is poured in funnel to a height above the lower level of the specimen in the sieve when the latter is placed within the funnel. At the end of one to six hours 25 to 50 ml. water are drawn off into a centrifuge tube and centrifuged. The supernatant fluid is pipetted off immediately and the sediment examined microscopically. A piece of ice placed on top of the specimen may speed up the migration of the larvae downward. The larvae may be inactivated by a few drops of 5 per cent sodium hydroxide or iodine solution. (*Caution:* Free-living roundworms in the soil are also concentrated by this method and must be distinguished from the parasitic forms.)

RECOVERY OF OVA: FROM SOIL. Ova are recovered from the soil in surveys when it is desired to determine the amount of soil pollution with *Ascaris* or *Trichuris* ova. Several methods have been developed; that of Headlee (1936) is probably the most practical.

Technic. Soil is scraped from the surface layer of a suspected area. Five to 10 Gm. of the soil are placed in a 50-ml. centrifuge tube and 10 ml. of 30 per cent antiformin solution are added. This is allowed to stand for one hour, during which it is stirred and shaken frequently. The tube is then filled with a solution of sodium dichromate (sp. gr. 1.35), shaken thoroughly, and centrifuged at 1000 revolutions for two minutes. The surface film is removed with the aid of a loop to a 15-ml. conical centrifuge tube and the tube is filled almost to the top with water. This tube is centrifuged; the supernatant fluid is pipetted off and the sediment examined microscopically.

FROM FURNITURE, WINDOW SILLS, MANTLES, ETC. *Enterobius vermicularis* ova can be recovered from many objects within the house of infected persons by the utilization of transparent scotch tape. This method is also applicable to the other helminths which depend upon the eggs of the worm reaching the mouth of man for the completion of the life cycle.

Serologic and Allied Tests for Helminthic Diseases. Nearly every important helminthic disease has had a serologic test designed for its diagnosis. These tests, however, are of practical value only for cases in which diagnosis by other methods is impractical at certain stages of the disease and in which the worms cause severe tissue damage, i.e., schistosomiasis, paragonimiasis, echinococcosis, cysticercosis, trichinosis, and filariasis. Serologic tests have also proved of value in the diagnosis of fascioliasis (Lavier and Stefanopoulo, 1944) and onchocerciasis (Bozicevich et al., 1947). The tests usually have a group type of reaction and consequently require careful interpretation. Often intradermal, precipitin, and complement-fixation tests are available for the same disease but only the most practical are recommended below. The technic of performing complement-fixation, precipitin, and intradermal tests is discussed under Serology (p. 279), and will not be described below.

TESTS FOR SCHISTOSOMIASIS: COMPLEMENT FIXATION. The *antigen* used in this test is an alcoholic extract of the larval stages of the blood flukes in heavily infected molluscan tissues.

The test is of value particularly in early cases before eggs are extruded and in late cases where tissue reaction prevents the passage of ova. It may also be used to diagnose unisexual infections. Williams (1947) has found this test very useful in detecting chronic or persisting infections.

EUGLOBULIN PRECIPITATION (SIA). This is not a true serologic test. (See p. 523 for description.) It must be carefully evaluated.

INTRADERMAL TEST. The *antigen* consists of saline extracts of triturated adult worms or heavily infected snail livers.

The test is of value only in diagnosis since the reaction remains positive after treatment. It is not so effective as the complement-fixation test at present but promises to become more useful. Gonzalez and Pratt (1944) believe this test to be the most valuable.

TESTS FOR PARAGONIMIASIS: COMPLEMENT FIXATION. The *antigen* is *Paragonimus westermani* adults macerated in physiological saline.

The test is of value in cases where eggs are not passed in the sputum or feces.

TESTS FOR ECHINOCOCCOSIS: INTRADERMAL TEST (CASONI). The *antigen* is a clear, sterile hydatid fluid aspirated from the cysts in sheep or other common hosts. Antigen made by the Dennis (1937) method is probably the most potent. A comprehensive summary of the various serologic tests used in the diagnosis of echinococcosis is given by Grana (1945).

The test is of value in all cases of echinococcosis but particularly so prior to operation. It is the test of choice in all cases except old complicated ones.

COMPLEMENT FIXATION. Purified powdered antigen prepared by Dennis technic is used. This is diluted 1 : 5000 and used as in the Kolmer modification of the Wassermann test.

The test is of value in old complicated cases, but otherwise, the intradermal test is superior.

TESTS FOR CYSTICERCOSIS: INTRADERMAL TEST. The *antigen* consists of fluid from cysticercus larvae of various species in domestic animals.

The test is of value in suspected cases. It should be very carefully evaluated and confirmed by the precipitin test. It is not as reliable as the tests for echinococcosis.

PRECIPITIN TEST. The same antigen is used as for the intradermal test just described.

The precipitin test is of value in confirming a diagnosis of cysticercosis made by the intradermal test.

TESTS FOR TRICHINOSIS: INTRADERMAL TEST. The *antigen* is most satisfactorily made from trichina larvae ground in distilled water, dried, and reground.

The test is of value after the twenty-first day of infection. Very heavy and very light infections are often missed. False positive and nonspecific reactions may occur. The reaction remains positive for years after recovery from the acute attacks.

PRECIPITIN TEST. The same antigen is used as for the intradermal test for trichinosis.

The test is of value after the fourth week of infection. It is of about the same specificity as the intradermal test.

TEST FOR FILARIASIS (DUE TO WUCHERERIA SPECIES): INTRADERMAL TEST. The *antigen* is prepared most commonly from *Dirofilaria immitis,* the dog heartworm. Washed, dried, lipoid-free worms are ground into a fine powder. Antigen made by the Bozicevich and Hutter (1944) method is probably the most potent. Hunter, Bozicevich, and Warren (1945) have shown that antigen prepared from the microfilariae of *W. bancrofti* is more specific but it is more difficult to obtain. Much work is being carried on at the present time on these tests. For the more recent results see Goodman et al., 1945; Oliver-Gonzalez and Morales, 1945; Augustine and Lherisson, 1946; Zarrow and Rifkin, 1946; Wharton and Stelma, 1946; Warren et al., 1946; Franks, 1946; Saunders et al., 1946.

The test is of value especially in detection of Wuchereria infection before microfilariae are present in the peripheral blood or when there is a light infection in which the embryos may never be found.

Preparation of Permanent Mounts of Adult Helminths. Excellent and striking permanent mounts can be made of many helminths, especially the flukes and tapeworms. The stains usually employed are carmine or hematoxylin solutions. However, each worker prefers his own technic and these are so detailed and varied that it is impossible to outline them here. A standard text on microscopic technic should be consulted by one desiring to make such preparations. A very practical and simple method which has given excellent results recently at the Naval Medical School is given below.

GLYCEROGEL METHOD (YETWIN, 1944). This is used for mounting small worms of all groups, larvae, and ova.

TECHNIC. Fifteen Gm. Bactogelatin (granular, Difco) are dissolved in 150 ml. boiling water. To this are added 50 ml. glycerin (reagent, Merck). After mixing thoroughly, 100 ml. of a 1 per cent chromium and potassium sulfate solution (C.P., granular, Merck) and 1 ml. liquid phenol (U.S.P.) are added. The medium jells at room temperature but liquefies when heated. The specimens are transferred directly from formalin or glycerin solutions to the mounting medium and allowed to cool. Slides are ready for study or class use in about 10 hours.

Examination of Intermediate Hosts for Helminth Larvae. Only the examination of the more important intermediate hosts is briefly outlined below.

SNAILS (FOR LARVAL TREMATODES): COLLECTION OF SNAILS. Great care must be exercised in collecting snails in areas where schistosomiasis is present. Since infection may result from contact with a snail from which cercariae are escaping or from contact with water containing cercariae, it is advisable to wear rubber gloves and boots. In areas where this disease is absent these precautions are unnecessary. A small net with a coarse mesh may be used to separate the snails from the mud. Mollusks which are resting in moss may be

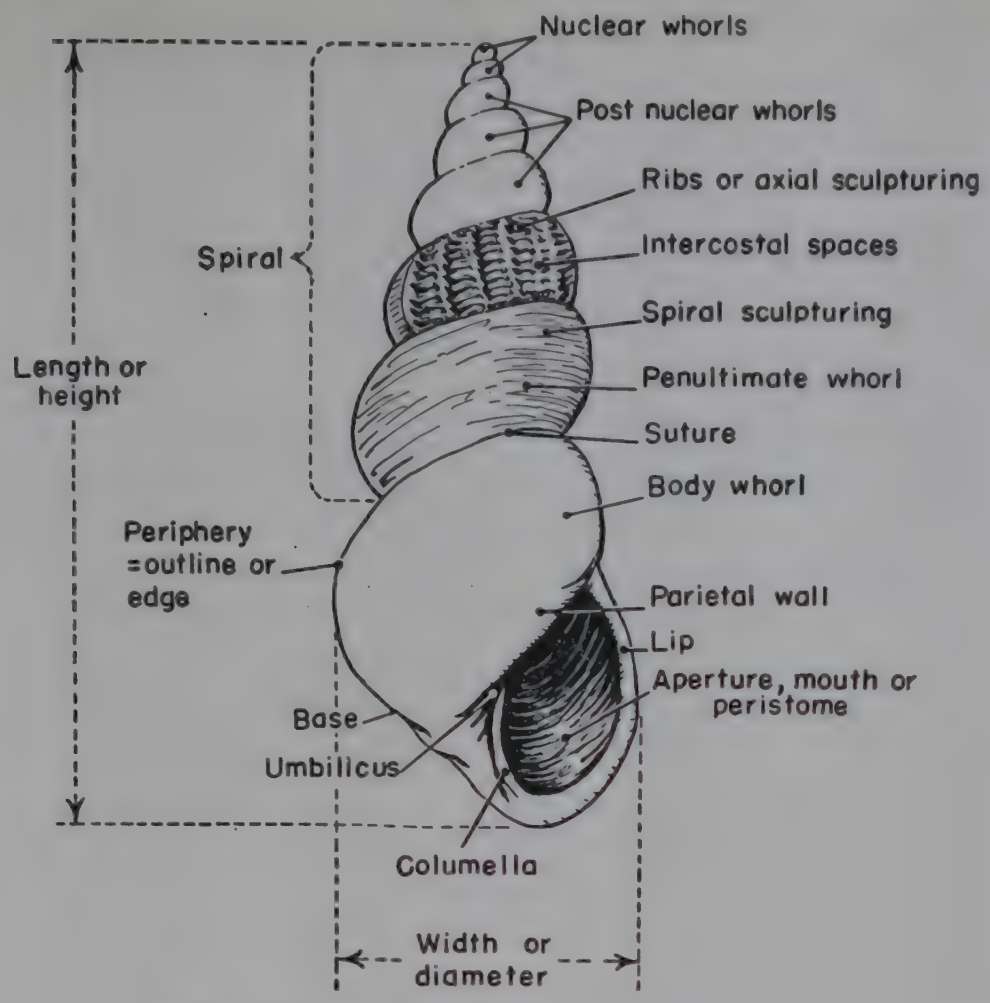


Diagram of snail to show identification characters.

obtained by shaking small quantities over a pan, screen, or pieces of burlap. Those which are resting on plants may be removed with forceps.

ISOLATION AND IDENTIFICATION OF CERCARIAE. Snails which are brought in the laboratory should be placed in half-pint bottles, in lots of about a dozen. A gauze top should be placed over the jar to prevent their escape. Each jar should be examined daily for emerged cercariae. It may be desirable to isolate the snails, once cercariae are demonstrated, in order to ascertain the source of cercarial production. Cercariae can be found by crushing the digestive gland located in the spire of the snail. The shells are usually quite delicate and if care is exercised they may be cracked without injury to the tissues within. The pieces should be removed and the digestive gland exposed, macerated in water, and examined microscopically. Cercariae obtained in this manner may be immature and unlike those escaping naturally; therefore this procedure should be used only if there is not sufficient time to await the normal emergence of cercariae. Each species of fluke parasitic in man has cercariae which may be identified by their structure. Insofar as possible, cercariae should be examined in the living state with the help of such stains as neutral red or Nile blue sulfate (1 : 1000). Those of the schistosomes are characterized by (1) the presence of a forked tail and (2) the absence of a pharynx; not all forked-tailed cercariae attack man.

IDENTIFICATION OF SNAILS. The correct identification of snails is difficult and should be referred to an expert whenever possible. The U. S. National Museum will classify material sent to it and return the identification to the collector. Living specimens should be preserved in 70 per cent alcohol, *never* in formalin, and shipped in vials. Large empty shells should be wrapped in paper. Collecting data should always be included; geographic and ecologic information are of particular importance. Snails can usually be identified by the form and markings of their shells and by the structure of the radula, a lingual ribbon

or filelike set of teeth placed in the buccal cavity of each animal. The lingual ribbon is built of thousands of tiny teeth of glasslike substance arranged in closely packed rows. Each row has seven teeth: one central tooth or rachidian bordered on both sides by three different teeth—the lateral or median, the inner marginal, and the outer marginal.

PREPARATION OF RADULA FOR MICROSCOPIC EXAMINATION. The radula is heated gently in strong potassium hydroxide and the flesh needled away. It is then washed in water, stained in mercurochrome, and transferred to 90, then 98 per cent alcohol, then xylol. It is mounted in Canada balsam on a slide. The teeth are broken apart with needles before the slide cover is applied. Enough variation in denticle counts has shown that radulae are helpful, but not final in identification.

MOSQUITOES AND OTHER DIPTEROUS INSECTS (FOR LARVAE OF FILARIAL WORMS): TECHNIC. Female mosquitoes or other dipterous insects are killed by chloroform, cyanide, or other method. Identical specimens are identified and preserved or mounted for confirmation. The wings and legs are removed. The abdomen is separated from the head and thorax and placed in separate drops of physiological saline on the same or different slides. The abdomen is teased and macerated with fine needles and the material is examined for

TEETH OR RADULAE OF SNAILS

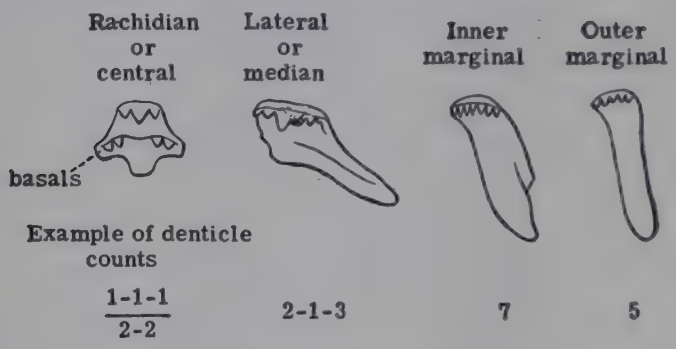


Diagram of radulae of snails.

microfilariae under the low-power objective of a compound microscope. The process is repeated with the head and thorax, search being made for advanced stages of larvae. All forms present should be counted and the age of the worms noted. For permanent record all positive preparations may be fixed with Schaudinn's or other fixative and stained with hematoxylin and eosin.

FOOD (FOR INFECTED LARVAL FORMS OF HELMINTHS). The most important foods containing infective stages of helminths are pork and pork products, beef and beef products, fish, crayfish, and vegetables. All of these foods except vegetables may be examined best by the methods discussed under autopsy and biopsy technics, i.e., teasing out the infective form in normal saline, compression, digestion, or sectioning. Pork is examined for trichina larvae and the cysticercus form of *Taenia solium*. Beef is investigated for the cysticercus of *Taenia saginata*. Fresh-water fish are inspected for the plerocercoids of the broad fish tapeworm and the metacercariae of certain liver and intestinal flukes; although salt-water fish may in certain cases carry infective forms of some flukes, as a general rule they can be absolved of any relation to helminthic disease in man. Crayfish in endemic areas carry the metacercariae of *Paragonimus* in their gills or muscles of the appendages.

Vegetables may act as the instrument of conveyance of the metacercariae of the sheep fluke and the giant liver fluke in the rather restricted endemic areas. They have a cosmopolitan importance, however, in helminths transmitted by ingestion of infective ova. Especially in areas where human feces are used as fertilizer, *Ascaris*, *Trichuris*, and other ova contaminate the vegetables. They may be removed by scrubbing and recovered by centrifugation or flotation.

CHAPTER 25

Medical Entomology

The first important contribution to the field of Medical Entomology came when Patrick Manson in 1878 discovered that *Culex quinquefasciatus* acted as the intermediate host and vector of *Wuchereria bancrofti*. Immediately upon the heels of this epochal contribution came other reports of various species of insects and arachnids that were suspected and subsequently proved to be vectors of many important human diseases. Ross in 1897 announced the discovery of malaria zygotes on the stomach wall of his "dappled-wing" mosquitoes; in 1880 Carlos Finlay suggested and propounded the theory that his "tiger" mosquito carried yellow fever; and in 1900 on the island of Cuba members of the United States Yellow Fever Commission proved beyond a doubt that *Aedes aegypti* was the vector of the disease under study. In the field of entomologic literature, Nuttall, in 1899, compiled available notes on "the role of insects, arachnids, and myriapods as carriers in the spread of bacterial and parasitic diseases of man and animals." Since that time, many excellent texts have been written on the role of arthropods as vectors of disease; and, today, *Medical Entomology* ranks with some of the older basic sciences in the field of Public Health.

With these notable advances clarifying the role of arthropods as vectors of disease, a remarkable change has taken place in the viewpoints of scientists and laymen regarding the importance of insects and arachnids to the health of man. A vital approach to a better understanding of the pertinent aspects of the epidemiology of many diseases is through a comprehensive study of the vectors involved. It follows, therefore, that in order to carry out preventive measures effectively, a study of the morphology and ecologic habits of the specific vector is fundamental.

Basically, the manner in which insects and their allies affect the health and well-being of man may be treated under the following headings:

1. By Transmitting Agents of Disease. Transmission takes place by mechanical or biologic means.

Mechanical Transmission. This is accomplished by agents acting as carriers or mechanical transporters of pathogenic organisms which undergo neither a developmental change nor a multiplication within the body of the arthropod. The agents are not essential to the life cycle of the parasite. Many nonbiting flies, in particular the house flies, gaining access to human food and feces, become important factors in the spread of typhoid, cholera, and bacillary and amebic dysentery.

Biologic Transmission. This is accomplished by agents acting as essential hosts and vectors of pathogenic organisms. In biologic transmission, the disease or-

ganisms within the body of the arthropod: (1) increase in numbers (plague), (2) undergo a developmental change without multiplication (filariasis), or (3) undergo a developmental change with multiplication (malaria).

2. By Invading Tissues. Many species of arthropods in either the adult or larval stage may play an important part in producing pathologic conditions. Thus, the larval stages of various diptera cause a disease commonly known as *myiasis*.



CONENOSE BUGS
transmit
Chagas' Disease



FLEAS
transmit
Plague
Endemic Typhus
Dog Tapeworm



LICE
transmit
Relapsing Fever
Epidemic Typhus Fever
Trench Fever
cause: Vagabond's disease



"SOFT" TICKS
transmit
Relapsing Fever
cause: Tick paralysis



"HARD" TICKS
transmit
Tick-Borne Rickettsioses
(Rocky Mt. Spotted Fever)
(Brazilian Spotted Fever)
(Fievre Boutonneuse)
(So. African Tick Fever)
("Q" Fever)
Tularemia
Colorado Tick Fever
Bullis Fever
cause: Tick paralysis



MITES
transmit
Tsutsugamushi
(Scrub Typhus)
cause: Dermatitis



NON-BITING FLIES
transmit
Yaws
Typhoid Fever
Dysenteries
Cholera
Conjunctivitis
cause: (larvae) Myiases



BITING FLIES
transmit
Tularemia
Sand-fly Fever
Filariases (Loaiasis, Onchocerciasis)
African Sleeping Sickness
Leishmaniases (Kala-azar, Oriental Sore)
Bartonellosis



MOSQUITOES
transmit
Malaria
Yellow Fever
Dengue
Filariasis (Elephantiasis)
Encephalitis

Types of arthropods transmitting human diseases (selected examples).

The chigoe (sandflea) and sarcoptic mite invade the tissues of man to cause disturbing lesions. The accidental invasion of ears, nose, and eyes by living insects may produce serious complications; fortunately this type of invasion is not a common occurrence.

3. By Inoculating Poisonous Substances. Inoculations may be closely related to mechanical effects, but in this instance the disturbance of functions of the human body is due to the chemical action of toxins produced by the arthropod. Their action may be local, systemic, or both. Toxins produced by the bites of certain species of ixodid or argasid ticks are known to produce paralytic symptoms in some individuals. Other arthropods—spiders, scorpions, and bees—may introduce venoms which give rise to general or local symptoms, sometimes followed by a condition of shock with fatal results. Bacterial infection of the skin frequently follows the scratching of mosquito and other insect bites. Allergic reactions may be caused by venoms, scales, hairs, or other products of arthropods not generally recognized as poisonous.

4. By Being Pests of Man. Many arthropods by virtue of their very presence interfere with man's health without transmitting or causing diseases or producing poisons that are noxious to the individual. The copra or coconut beetle of tropical areas is extremely annoying since the adults, attracted by lights, enter screened or unscreened quarters and crawl over the body and into the hair of the occupant. The annoyance and worry produced by the mere presence of insects and spiders may cause nervous disorders and sensory hallucinations.

The Phylum Arthropoda

Classification and Morphology. No other group of the Animal Kingdom approaches the arthropods in number of individuals. There are more known species in this phylum than in all other phyla combined; the estimated total in insects alone is more than a million. As would be expected, this enormous group exhibits great diversity of habit and structure. Although the phylum includes forms which are very unlike in appearance, the typical arthropod is well defined by: (1) a general absence of cilia; (2) a body cavity, the hemocele, which is in free contact with the circulatory system; (3) bilateral symmetry; (4) paired, jointed appendages arising from the body segments of the adults and of some immature forms; (5) the mouth and anus at opposite ends of the body; (6) dorsal circulatory system and ventral nervous system; and (7) body covered with a hard chitinous exoskeleton.

Several distinct groups of arthropods can be recognized, and the members of these groups will be discussed under the following classes:

Class Crustacea (crabs, lobsters, crayfish)
Class Chilopoda (centipedes)
Class Diplopoda (millipedes)
Class Arachnida (mites, ticks, spiders)
Class Insecta (Hexapoda) (flies, bugs, lice, fleas, mosquitoes, etc.)

Crabs, Lobsters, Crayfish, Water Fleas (Class Crustacea)

Cyclops and *Diaptomus,* commonly known as water fleas or copepods, are involved in the life cycles of the fish tapeworm, *Diphyllobothrium latum,* and the guinea worm, *Dracunculus medinensis.* The larger crustaceans—crabs, lobsters, and crayfish—act as intermediate hosts for the lung fluke, *Paragonimus westermani.* Metacercariae were found by Tubangui (1946) in the uncooked leg muscles of crabs belonging to the genus *Potamon* in the Philippines. Most of the members of this class and all medically important forms are aquatic.



Cyclops containing larvae of *Dracunculus medinensis.* (Courtesy, Strong: Stitt's Diagnosis, Prevention and Treatment of Tropical Diseases, 7th ed., Philadelphia, The Blakiston Company.)

Control measures directed against the crustacean intermediate hosts of these parasites are not practical. Prophylaxis consists in safeguarding the water supply to avoid ingestion of copepods infected with the guinea worm. Shellfish likely to harbor the infective stage of the lung fluke must be thoroughly cooked before being eaten.

Centipedes (Class Chilopoda)

Bites from the small centipedes found in temperate climates rarely give rise to more than local symptoms. The large species encountered in the Tropics inflict painful bites which may result in disability for several days. Individuals are usually bitten while sleeping or when putting on shoes or clothing in which the centipede has hidden. The various species usually live in moist, protected places and are largely nocturnal in habit. During the day they may be found under the loose bark of dead trees or under logs, boards, stones, leaves, and other debris on the ground. In handling such material, there is some chance of grasping a centipede accidentally, in which case it will bite in self-defense.

The centipedes are elongated, flattened arthropods with one pair of legs arising from each segment of the body; they have one pair of antennae; wings are absent.

Poison glands are located at the base of the first pair of legs, modified to function as mouth parts.

Control measures, other than caution, are usually not necessary.

Millipedes (Class Diplopoda)

The millipedes are inoffensive and harmless except in the rare case when they act as intermediate host for the rat tapeworm, *Hymenolepis diminuta*. A large species common in the Fiji Islands is known to spray a fluid from special glands in the body which may produce a dermatitis upon contact with skin or mucous membranes. Millipedes have two pairs of legs arising from each apparent body segment except the first four. The body is typically round in cross section. These myriapods live in a moist environment, feeding primarily on decaying vegetable matter.

Burtt (1947) emphasizes the injurious effects of the secretions of millipedes and classifies them into two categories: (1) caustic action on the skin as a result of immediate contact with the millipede, and (2) injury to sight as a result of squirting fluid from the repugnatorial foramina.

Ticks, Mites, Spiders (Class Arachnida)

Since ticks and mites are involved in the transmission or cause of several important human diseases, members of this class will be discussed separately in Chapter 26.

Insects (Class Insecta—Hexapoda)

This class includes the great majority of arthropod species of medical importance and will be taken up in detail in Chapter 27.

CHAPTER 26

Scorpions, Spiders, Ticks, Mites (Class Arachnida)

The arachnids constitute one of the two important classes of the phylum Arthropoda. They are cosmopolitan in distribution and many forms are known transmitters of various diseases of man and animals in addition to being serious human parasites. A member of this class, *Boöphilus annulatus,* was the first arthropod shown to be a vector of a protozoan disease, for Smith and Kilborne proved in 1889 that Texas cattle fever was transmitted from animal to animal by this tick, the pathogenic organism passing in an hereditary manner from adult through the egg to the next generation. The ticks and their close relatives, the mites, have since been incriminated in the transmission of various human diseases.

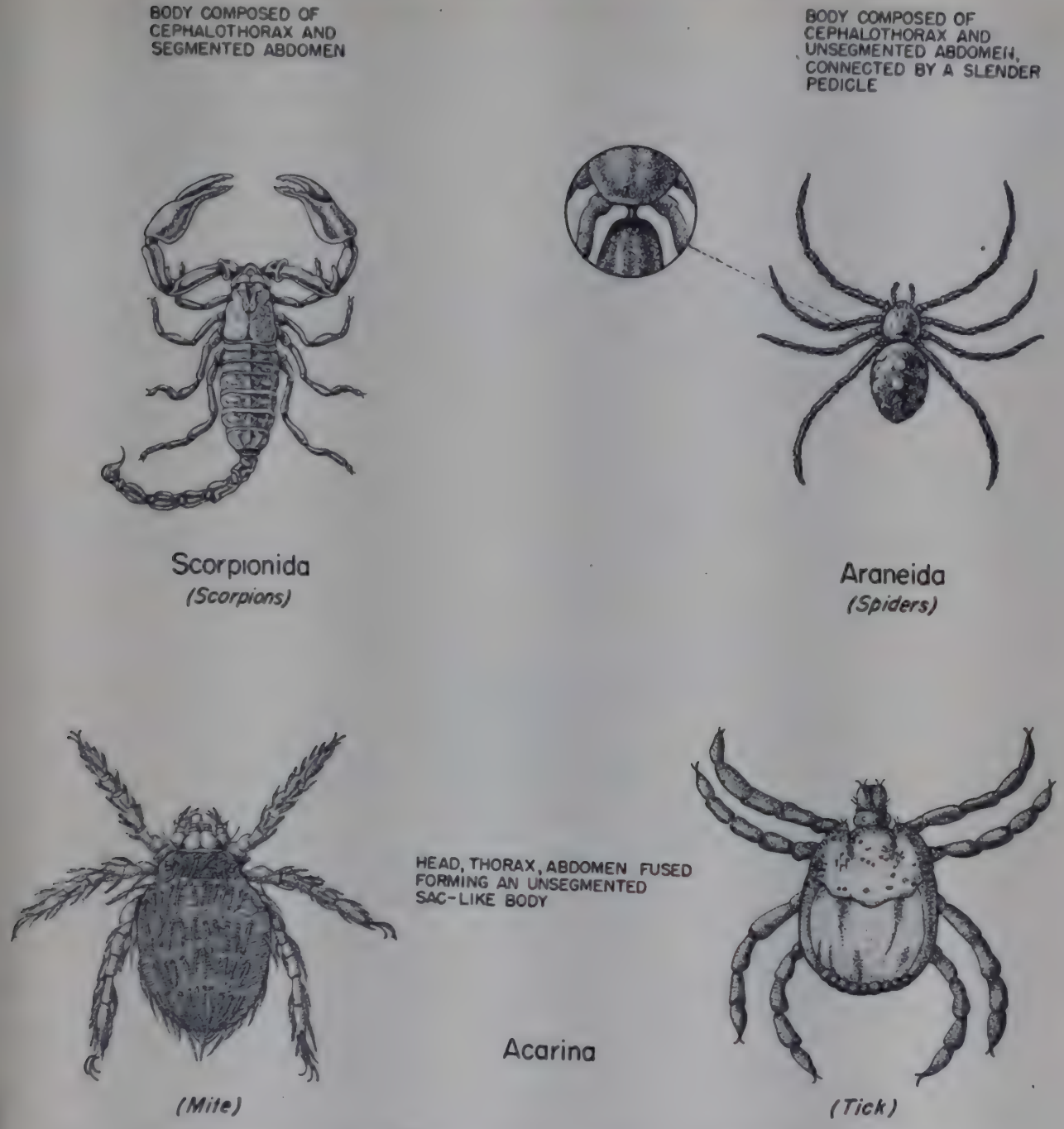
Typical forms of arachnids are air-breathers. The head and thorax are fused to form a cephalothorax. Wings and true antennae are lacking. The adults and nymphs have four pairs of walking legs; the larvae, only three.

The class Arachnida is divided into many orders, only three of which are of primary importance:

Order Scorpionida (true scorpions)
Order Araneida (spiders)
Order Acarina (ticks and mites)

True Scorpions (Order Scorpionida)

Scorpions have formidable claws or pedipalps with which they seize their prey. The last abdominal segment terminates in a ventrally curved spine and carries the poison gland, the sting curving downward when the tail-like post-abdomen is extended. When the animal is disturbed or poised for an attack this structure is flexed upward and forward. The victim is struck quickly and repeatedly by a downward movement of the spine. Although scorpions transmit no disease, the larger forms are to be avoided. The importance of scorpion poisoning has been emphasized recently by Sergent (1938) in North Africa, Baser (1939) in India, Faust (1940) in northern Mexico, and Shulov (1942) in Palestine. The studies of Waterman (1938) emphasized the importance of poisoning by scorpions in Trinidad, where, however, only the severe cases came under medical observation. The effects of the sting of small species found in southern United States and California are probably never fatal, although they may be quite painful and produce mild symptoms of poisoning. Like the centipedes, scorpions are predaceous in habit and may be considered beneficial as a group. The scorpions of temperate climates are usually small, but those of the Tropics attain lengths of 7 inches or more. They are usually found under stones, leaves, and bark and in the axils of coconut and



Key characters for identifying orders of arachnids of medical importance.

banana trees. During rainy seasons the smaller species commonly enter houses to hide in shoes, clothing, towels, or beds. General extermination is impractical.

Certain arachnids superficially resembling true scorpions are particularly common in Florida, Texas, and California, being known by such common names as whip scorpion, vinegaroon, or mule killer. They are often regarded as being poisonous, although they have no stinging apparatus such as is found in the true scorpions. They may bite, but the effect is no more than that of a slight pin-prick. The distinguishing characteristic is the slender, whiplike appendage on the last abdominal segment.

Spiders (Order Araneida)

Spiders are universally feared although many are harmless, their bite producing a slight stinging sensation with perhaps some local redness. The bite of only a

few species is dangerous to man. In the United States, the "black-widow," "hour-glass," or "shoe-button" spider has been held responsible for symptoms of poisoning in about 400 cases, with a record of several deaths. The pain resulting from the poison may be excruciating, the victim scarcely responding to morphine. The venom, like that of the cobra, appears to be a toxalbumin and its most damaging action is on nerve endings.



Latrodectus mactans, female (black widow). Ventral surface showing orange-red hour-glass spot. (Original.) Approximately actual size.

Spiders can be recognized by their distinct cephalothorax bearing two chelicerae and two six-segmented, leglike pedipalps. The abdomen is rounded, soft, and unsegmented and is connected to the cephalothorax by means of a narrow pedicel. The legs consist of seven segments, the tarsi bear two or three claws. The modified pedipalps of the male serve to transfer the spermatozoa to the female at the time of mating. Three or four spinnerets for the production of spiders' "silk" are located near the tip of the abdomen on the ventral side.

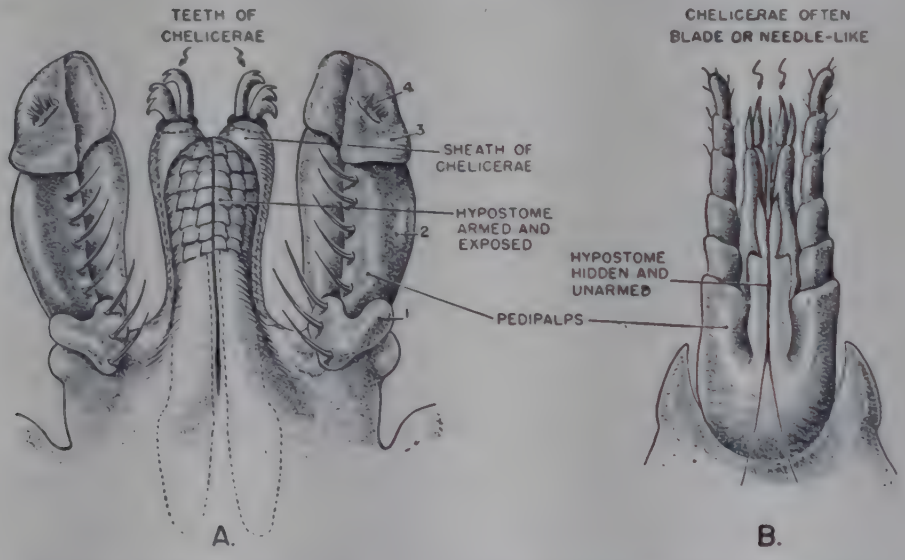
There are more than 30 families of spiders in the order Araneida. The "black widows" belong to the genus *Latrodectus* (family Theridiidae). The females are coal-black with a characteristic orange-red spot on the underside of the globular abdomen. There may also be some red or yellow markings on the upper side o

the abdomen, particularly in the male. Herms gives an excellent description of this spider in his text, *Medical Entomology*. The term "tarantula" is loosely applied to any large spider but is related specifically to members of the superfamily Aricularoidea including the large hairy trap-door spiders in southern United States (family Lycosidae), the bites of which cause no great discomfort.

Fly sprays are not effective against black-widow spiders. Ten per cent solution of DDT in kerosene applied to the webs was demonstrated by Van Riper (1946) to be lethal to the spiders, the effect of the spraying lasting for several days. Creosote acts as a satisfactory repellent and it may be sprayed under seats of privies and into corners of basements with good effect. Egg sacs may be brushed down and crushed or burned.

Ticks and Mites (Order Acarina)

The ticks and mites are well known for their ability to transmit several important diseases such as Rocky Mountain spotted fever, relapsing fever, and scrub



Mouth parts of: (A) ticks; (B) mites. Ventral aspect (schematic).

typhus; they may also be semiparasitic on man and animals without carrying disease organisms. The majority of the acarids are round or oval forms with head, thorax, and abdomen fused to form a saclike unsegmented body. Members of the order undergo a marked metamorphosis in that the adults and nymphs possess four pairs of walking legs while the larvae have only three. Wings and antennae are absent, and eyes may or may not be present.

The *mites* are usually small, many being microscopic. The body is membranous in appearance and often clothed with long hairs; the hypostome is hidden and unarmed; the palpi never have more than five segments, the last segment being provided with sensory hairs. The *ticks* are larger forms and are mostly macroscopic. The body is sclerotized or leathery in appearance and hairs, if present, are short. The hypostome is exposed and armed with strong cutting teeth; the palpi are four-jointed.

The important groups of the Acarina will be discussed subsequently in greater detail.

Mites

The mites represent a large proportion of the order Acarina and one may not be aware of the large number of species occurring within this group because of their microscopic size. Many are free-living and a number of them may be accidental parasites of man. The life histories of mites show much variation; typically there are four stages: egg, larva, nymph, and adult. Some mites are known to be viviparous and others, ovoviviparous; members of the genus *Pediculoides* bring forth adult males and females. There are six families of mites that contain species of medical importance:

Family Trombiculidae (chiggers)
Family Sarcoptidae (itch mites)
Family Demodecidae (hair-follicle mites)
Family Tarsonemidae (louse mites)
Family Parasitidae (tropical rat mites)
Family Tyroglyphidae (cheese mites)

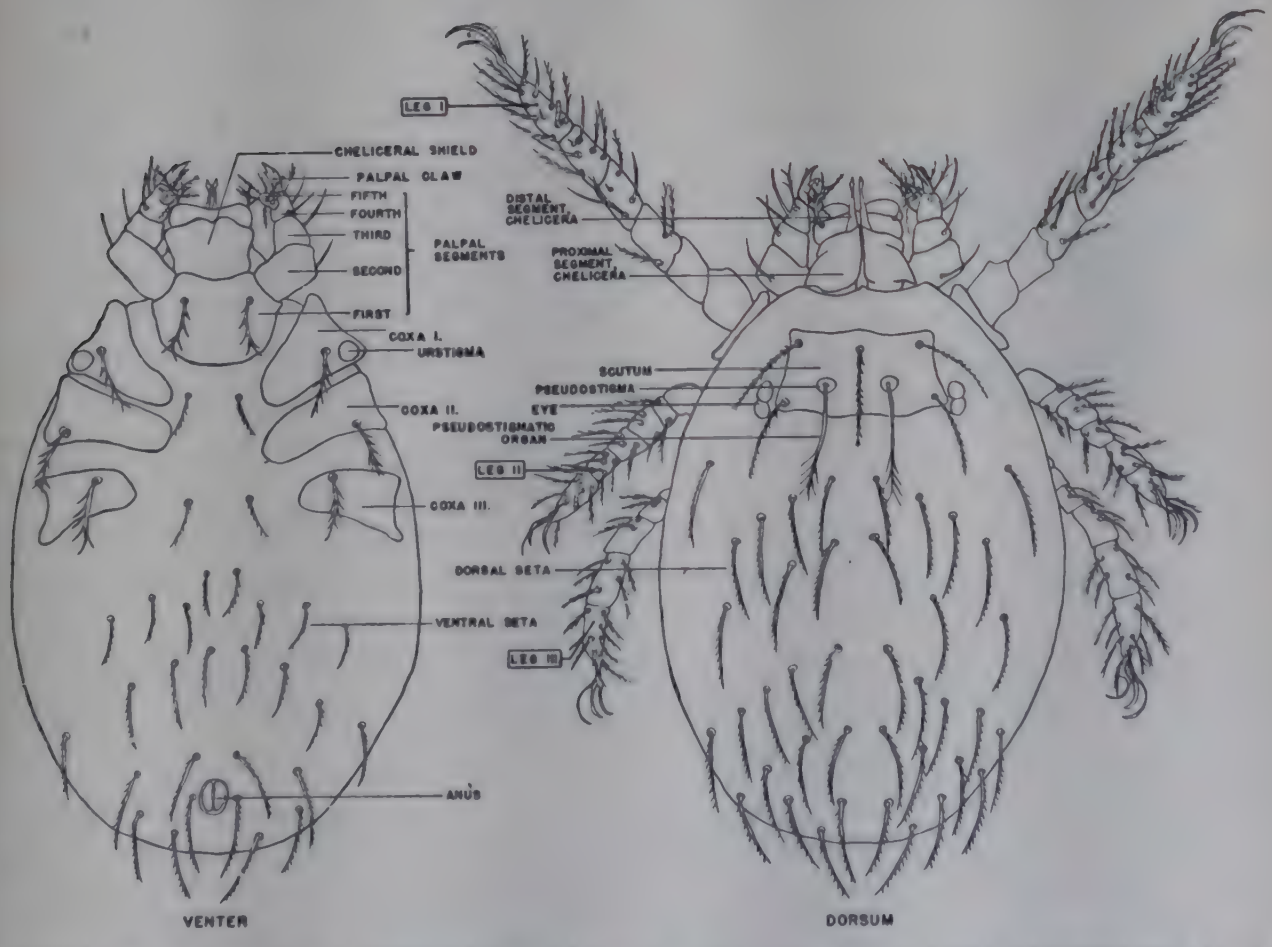
Chiggers, Redbugs (Family Trombiculidae). A form of typhus fever, commonly known as tsutsugamushi fever, mite or scrub typhus, is transmitted by the larval forms of species of the genus *Trombicula.* Only two species of chiggers are known to carry the disease: *Trombicula deliensis* and *T. akamushi. Trombicula walchi,* thought to be the vector in New Guinea, is probably only a local variant of *T. deliensis* (Wharton and Johnson, 1946). *Trombicula akamushi* is the important vector in Japan and Formosa. Chiggers are well known as causing a severe dermatitis (chigger or redbug dermatitis) due to a specific poison secreted by the mites at the time of feeding. Inflammation in the form of red blotches may not be noticed until 12 to 24 hours after initial attachment of the parasites; after a day or so these blotches become blisters and scab over. Secondary infection due to scratching may give rise to a variety of conditions, and Toomey (1921) reports that persons going barefooted in chigger areas in the Ozark Mountains have lost toes as the result of secondary infection from chigger attacks over the joints. In the Treasury Islands (upper Solomons), Perry reported that 45 per cent of treatments at one base dispensary were for small leg ulcers resulting from infections started by scratching the site of the bites of *Schöngastia blestowei.* New arrivals in infested areas may suffer severely for a season or two, but are said to appear to build up a tolerance or perhaps an immunity to bites. These mites do not burrow under the skin but attach themselves singly on man, inside the rim of the hair follicles. They are detached by scratching or by the rubbing of clothing against the body. *Eutrombicula alfreddugesi* is the mite responsible for scrub itch in the southern part of the United States.

Wharton (1946) adequately discusses the vectors of tsutsugamushi disease and as a result of extensive field surveys in the Pacific Ocean areas states that the rickettsia has been recovered only from mites of the tsutsugamushi group, and in areas where the disease does not occur mites of this group have not been found:

where trombiculid faunas were demonstrated and well known, tsutsugamushi disease was prevalent.

Biology and Morphology. Chiggers are known to parasitize all groups of land vertebrates—amphibians, reptiles, birds, and mammals. The larvae are small, about 0.3 mm. long, roughly egg-shaped, and bright red to cream in color. Upon repletion they drop to the ground where they pass into a quiescent state before transforming into nymphs. These are usually hourglass-shaped, have four pairs of legs, and are densely covered with hairs. The larvae and nymphs are sensitive to extreme dryness and are usually found in the upper layers of moist, well-drained soil. The adults of only a few species have been identified. Their food habits are largely unknown, but they probably feed on feces and body fluids of other arthropods or on decaying vegetation. The adults and nymphs of *Neoschöngastia indica* feed readily on mosquito and other insect eggs when confined to the laboratory. The eggs of trombiculids are apparently laid in clusters in the soil or other habitat favored by the adult. The chigger is the only parasitic stage in the life cycle of the mite, and dissemination is accomplished as a result of the movements of hosts to which they are attached. Colonies of chiggers will appear as reddish patches. On mammals, the chiggers will be found most frequently on and in the ears, about the groins, axillae, and mammae. Birds are parasitized on the legs and about the ears and eyes. On reptiles, chiggers are found between and under the scales.

Control. Clearing, oiling, and burning areas are good preventive measures used



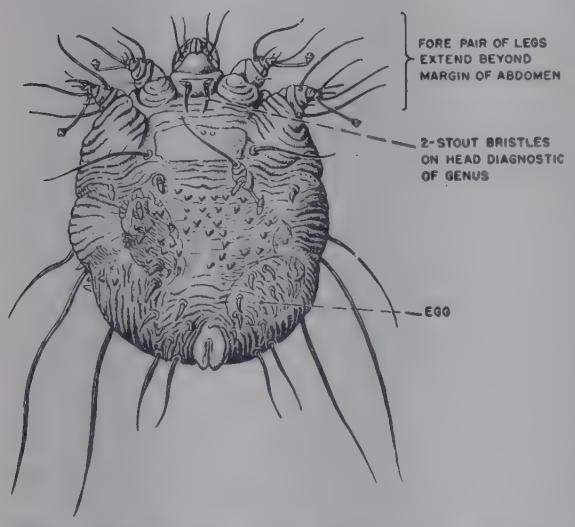
Dorsal and ventral views of *Trombicula akamushi*. (Courtesy, Lts. Johnson and Wharton, *U. S. Naval Medical Bulletin*, 46:459–472, March, 1946.)

to destroy the existing populations of rats and chiggers and eliminate the habitats favorable for reinvasion of the area. Methods of control are directed at preventing chiggers from biting man; this is accomplished by (1) avoidance of infective areas; (2) use of miticides on the skin and clothing—sulfur, dimethyl phthalate, dibutyl phthalate, and benzyl benzoate; (3) destruction of chiggers and their normal hosts; and (4) elimination of habitats suitable for chiggers and their hosts. DDT, because of its slow action, should be used against chiggers only as an area decontaminant. Five to 10 pounds of a 10 per cent dust per acre are required. Rats should be exterminated by large-scale and persistent trapping procedures.

Itch Mites, Scab Mites (Family Sarcoptidae). Members of one genus (*Sarcoptes*) frequently infest man, producing a skin disease called *scabies*. The several varieties are not host specific, and occasionally dairymen, sheep- and goat-herders, and children who fondle dogs and cats become infested with species normally parasitic on these animals.

Biology and Morphology. The human itch mite, *Sarcoptes scabiei* var. *hominis*, is an oval parasite; the male is 250 × 150μ, the female, about 400 × 300μ. The third and fourth pairs of legs in the female have bristles; the fourth pair in the male has suckers.

The female becomes mature in about two weeks and burrows into the skin, especially between the fingers, on the wrists, and around the genitals; any part of the body of infants may be affected. The tunnels produced by the mites are from 2 to 12 mm. long and tend to zigzag; they are dark gray, and the accumulation of feces makes a minute dirty papule at the entrance to the burrow. A vesicular elevation marks the location of the female at the blind end of the tunnel; scattered behind her are feces, eggs, and larvae, the eggs being next to the mother and the more mature young at the entrance to the gallery. A diagnosis can be made by demonstrating either eggs or larvae.



Dorsal view of female *Sarcoptes scabiei*.

Infection results from the passage of male and female mites or of an impregnated female from an infected to a healthy individual. Gerlach has demonstrated that infection may occur from parasites in bedding, towels, or other articles that come into contact with the skin. Chandler (1940) reports an epidemic of scabies following the use of an infected wrestling mat in a gymnasium.

Hair-follicle Mite (Family Demodecidae). *Demodex folliculorum* is a vermiform acarid about 400μ long, living head downward in the sebaceous glands of the nose and forehead. This mite seems to cause no ill effects in man, but the comedo may persist until the parasite is expelled. A related species causes a severe mange in dogs.

Louse Mite, Grain Itch Mite (Family Tarsonemidae). *Pediculoides ventricosus* is the well-known representative of this family. The parasites live on various insect larvae found in cereals, straw, and cotton and are most troublesome to laborers in the central grain-growing states. Man may develop a violent dermatitis from handling grain, beans, or cotton, or by sleeping on infested bedding material. The eruption with wheals, papules, and vesicles may cover the entire body and will appear within a day after exposure. Marked itching, burning, and sometimes fever accompany the dermatitis.

Chicken Mite, Tropical Rat Mite (Family Parasitidae). The chicken mite, *Dermanyssus gallinae,* infests poultry and chicken houses. Poultrymen may be troubled with a type of dermatitis similar to scabies, resulting from the bites of these parasites. The tropical rat mite, *Liponyssus bacoti,* readily attacks man, and it has been incriminated as a vector of endemic typhus in southern United States. This species also produces a dermatitis.

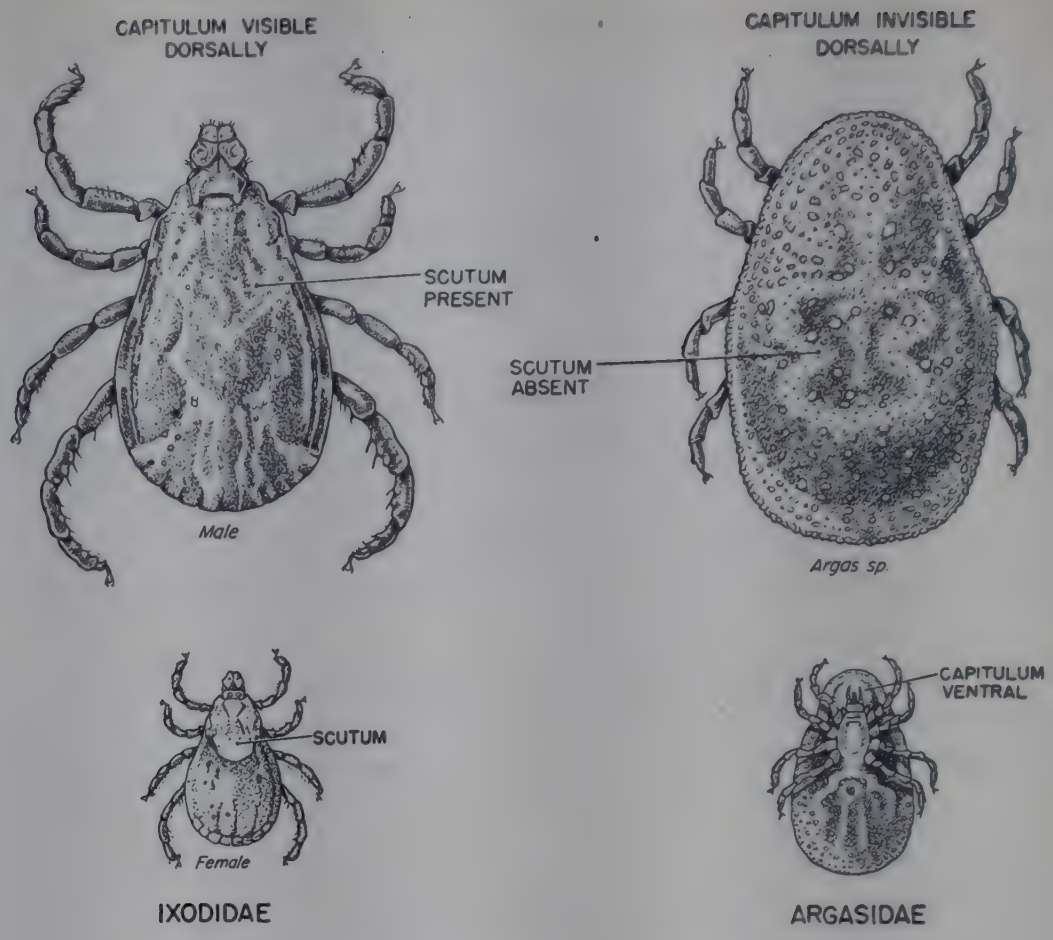
Cheese Mite, Copra Itch-mite (Family Tyroglyphidae). Mites of this family live on cheese, flour, and dried fruits and have been associated with human intestinal disturbances. Species of *Tyroglyphus* have been held responsible for "vanillism" in those who work with vanilla pods and for "copra itch" among copra workers. In many parts of the world the dermatitis consisting of an itching urticaria is known as "grocer's" or "baker's" itch. The mites of a species of *Glyciphagus* are frequently found in sugar and are the cause of this "grocer's" itch. *Rhizoglyphus parasiticus* is reported to be the cause of an itchlike infection of the feet of coolies on tea plantations.

Ticks

Ticks transmit a wide variety of pathogenic organisms including spirochetes, viruses, rickettsiae, bacteria, and toxins of unknown nature.

Classification and Morphology. Ticks differ from other acarids in having a median probe-shaped puncturing organ, the *hypostome,* which is beset with numerous teeth projecting backward, and in possessing *stigmal plates* in the body. The microscopic structure of the stigmal plates has been shown by Stiles to be of great value in differentiating the various species, particularly of the genus *Dermacentor.* The stigmal orifice, the opening of the tracheal system, is in the center of the plate. The false head, *capitulum* or rostrum, bears the *hypostome,* a pair of chitinous structures, the *chelicerae,* and a pair of segmented *pedipalps.* The genital opening in the adult is near the anterior part of the ventral surface; the anus is centrally located near the posterior third. The legs have six segments, the coxa being flattened on the surface of the body. The terminal tarsus ends with a pair of hooks and, at times, with a pulvillus. The nymph has stigmal plates but no genital aperture.

The ticks are divided into two families, the *Ixodidae* (hard ticks) and the *Argasidae* (soft ticks). They may be separated on the basis of several prominent external structures, particularly the shape and location of the capitulum and the presence or absence of a *scutum.* In the Ixodidae it is a smooth, hard, shieldlike



Family differentiation of ticks.

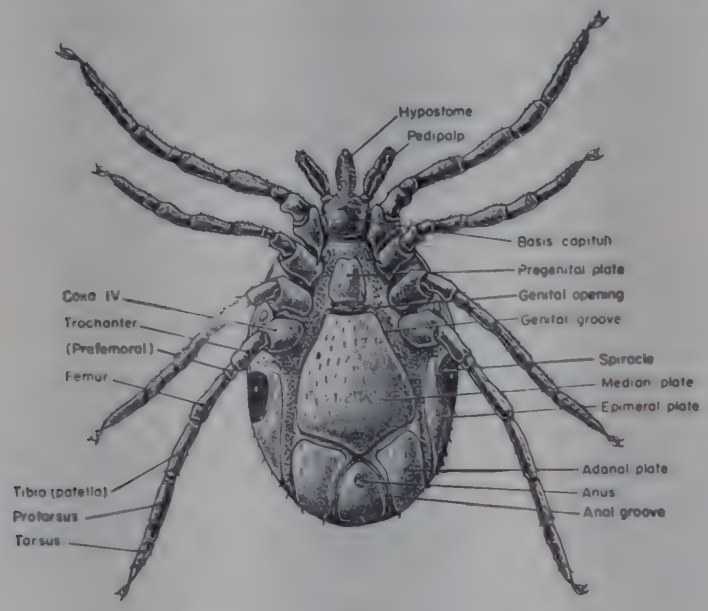
structure on the dorsal surface of the body. It covers almost the entire back of the male but only a small anterior portion of the female; thus the name "hard tick." This shield is lacking in the Argasidae; the apparently soft-textured body exhibits little protection, and the name "soft tick" is given to members of the group. The capitulum of the hard tick extends beyond the margin of the body and can be viewed dorsally; in the soft tick, it is ventral and generally does not extend beyond the anterior margin of the body. The stigmal plates are located posterior to each hind leg in the Ixodidae and between the third and fourth pairs of legs in the Argasidae.

Hard Ticks (Family Ixodidae). This family consists of about 10 genera, four of which are of special importance to man because of their disease relationships; *Amblyomma, Dermacentor, Haemaphysalis,* and *Ixodes* contain species that serve as reservoirs of infection or as vectors of diseases of man and animals. *Dermacentor andersoni,* the western wood tick, is the most important North American species, for it is known to transmit Rocky Mountain spotted fever, tularemia, and Colorado tick fever in the western states and is a suspected vector of Q fever. This species also causes tick paralysis, an acute intoxication usually caused by the bites of certain ixodids. If the tick or ticks are discovered and removed promptly, recovery occurs in a few days; otherwise, death may follow from paralysis of the respiratory system.

Amblyomma cajennense is a vector of São Paulo (Brazil) fever and a potential vector of Rocky Mountain spotted fever. It is a common species in Texas, Mexico,

and Central and South America. *Amblyomma hebraeum* transmits the rickettsia of South African tick-bite fever; *A. americanum,* the lone-star tick, is known to transmit tularemia and Rocky Mountain spotted fever. Recently, nymphs were reported naturally infected with the rickettsiae of American Q fever. Bishopp (1944) reports that this species probably plays a greater role in disease transmission than is generally recognized and should be looked upon with suspicion wherever encountered. Several other species of the genus are potential vectors of Australian Q fever and Rocky Mountain spotted fever.

Species of *Haemaphysalis* rarely bite man; however, members of this genus serve as important vectors of Rocky Mountain spotted fever and tularemia among



Ventral view of a hard tick.

rabbits which, in turn, are hosts of the larvae of *Dermacentor andersoni* and *D. variabilis* known to attack man readily when these ticks become adults. *Haemaphysalis humerosa* is believed to be a vector of Australian Q fever.

Ixodes species bite man freely, and several are known to cause tick paralysis. *Ixodes persulcatus* is a vector and reservoir of forest-spring encephalitis in the wooded areas of the Ural and Siberian regions.

Several species of ticks have been shown to harbor the virus of yellow fever from 4 to 28 days, although there is no evidence as yet of natural transmission.

Biology and Classification. The life history of hard ticks varies greatly depending upon the species. That of *Dermacentor andersoni* may be taken as representative of the group. Upon reaching a suitable host, the adults engorge, mate, and then drop to the ground. Males generally die at once; the females die after depositing their eggs in some protected place away from the host. From 200 to 8000 eggs are usually deposited in the course of a month; occasionally up to 20,000 in certain of the Ixodidae. After a period of development—a month or more—the small, six-legged larvae ("seed ticks") emerge. These larvae crawl up a blade of grass and wait until they can attach to some passing animal; they engorge and drop to the ground within a few days. After undergoing further development for several weeks, molting occurs and eight-legged nymphs appear; they in turn

climb up a blade of grass or a twig and await another passing animal (the second host). If fortunate in reaching one, they engorge, drop to the ground, molt after several weeks' development, and become mature adults.

The length of the life cycle varies greatly with the species and with weather conditions. *Dermacentor andersoni* requires two years, passing the first winter as an unfed nymph and the second winter as an unfed adult. Other species may complete the cycle in a single season; winter may be passed in the egg stage. The extraordinary capacity of the ticks to survive starvation compensates in part for the uncertainty of and frequent delay in reaching a new host. Larvae have survived seven to eight months and adults three to five years without food. Most hard ticks have either two or three hosts during their development; one or two species have a single host. Larvae and nymphs engorge on numerous species of small animals; adults commonly feed on large animals and readily attach to man.

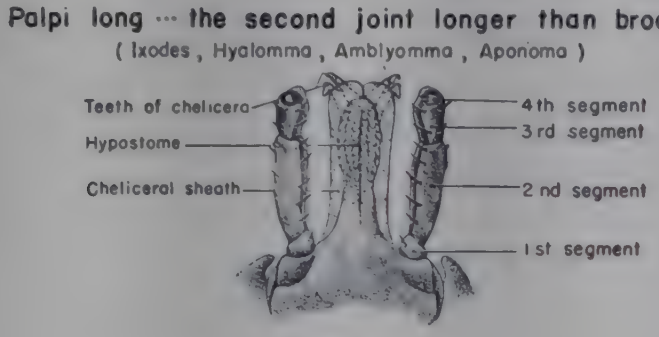
Key to Genera of Some Medically Important Hard Ticks
(Modified from Root and Huff)

1. Anal groove running in front of anus; pedipalps usually spatulate in form; second segment longer than broad; male with numerous ventral plates ..... *Ixodes*
   Anal groove either running behind anus or so indistinct that it cannot be seen clearly 2
2. Mouth parts nearly as long as basis capituli; second segment of pedipalps not much longer than wide 3
   Mouth parts longer than basis capituli; second segment of pedipalps longer than wide 8
3. Anal groove plainly visible; festoons usually present 4
   Anal groove absent or indistinct; festoons absent 7
4. Second segment of pedipalps with a well-marked lateral projection; eyes absent ..... *Haemaphysalis*
   Second segment of pedipalps not laterally produced; eyes present 5
5. Basis capituli quadrilateral in dorsal view; scutum usually ornate; male without ventral plates; fourth coxa of male larger than the others ..... *Dermacentor*
   Basis capituli hexagonal in dorsal view; scutum usually not ornate 6
6. Male without adanal plates and with fourth coxa larger than the others ..... *Rhipicentor*
   Male with adanal plates and with fourth coxa not much larger than the others ..... *Rhipicephalus*
7. Male with forked preanal plate; joints of fourth pair of legs greatly swollen ..... *Margaropus*
   Male with paired adanal and accessory plates; joints of fourth pair of legs normal ..... *Boöphilus*
8. Eyes absent; males without anal plates ..... *Aponomma*
   Eyes present .....
9. Eyes submarginal; males with adanal plates ..... *Hyalomma*
   Eyes marginal; males without adanal plates ..... *Amblyomma*

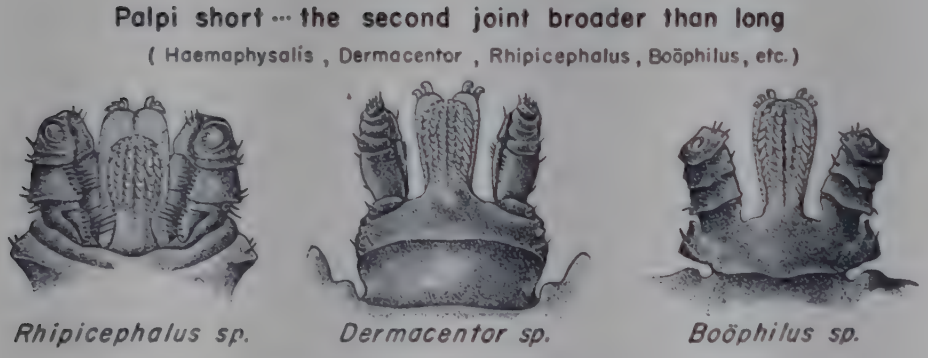
Control. The control of ticks with generalized host habits, long life, and wide distribution is a difficult matter. Dipping cattle and systematic grazing have been successful in localized areas. Clearing and burning underbrush and cutting grass close to the ground, especially near habitations, camps, and walks, greatly reduce

the chances of human infestation. Derris powder applied to animals at intervals of two to three days will substantially reduce the number of attached ticks.

Heavily infested regions should be avoided in the spring and early summer months. Frequent examination of the body, especially the head, is important in order to remove ticks before they have been attached for a long time. Insect repellents seem to have little effect against the adults. Ten per cent DDT dust applied to dogs and cattle give fair control, although the poison kills ticks slowly. It



Ixodes sp.



Rhipicephalus sp. *Dermacentor sp.* *Boöphilus sp.*

Ventral aspect of the capitula of some hard ticks.

should be used on cats with caution, since they may ingest sufficient amounts to become ill. DDT-oil sprays should never be used on animals over long periods of time.

Removal of Ticks and Treatment of Bites. Contrary to popular belief, a tick cannot be "unscrewed" to remove it from the host. The recurved teeth of the hypostome anchor it so firmly that the capitulum is torn off and left in the wound; or a fragment of skin is torn out with it when the tick is carelessly pulled off, thus producing severe irritation. This is especially true of ticks belonging to the genera *Amblyomma* and *Ixodes*. To dislodge a tick without leaving the hypostome, its body is grasped with a pair of forceps close to the site of attachment, and the tick removed by gentle traction; iodine or similar disinfectant is applied to the wound.

Soft Ticks (Family Argasidae). Members of the genus *Ornithodoros* are important vectors of relapsing fever. *Ornithodoros moubata* (the tampan) is the common vector of African relapsing fever. Both nymphs and adults transmit the infection by contaminating the bite with coxal fluid. These ticks infest native huts, particularly the rest houses along the routes of travel. They hide during the day in crevices of floors and walls and appear at night to feed. Several species of *Ornitho-*

doros occur in the United States; *O. coriaceus,* the "pajaroella" of the southwestern United States and Mexico, is regarded by Herms as one of the most venomous of ticks; it produces a small wound which does not heal for several days. *Ornithodoros talaje* is the important vector of relapsing fever in Mexico and Panama; *O. turicata* in Texas, Kansas, and New Mexico; and *O. hermsi* in California, Colorado, Washington, Idaho, and Nevada. Our knowledge of the latter's distribution is fragmentary, but Herms reports it as common in the Sierra Nevada and San Bernardino mountain ranges at elevations of 5000 to 8000 feet. It appears to be typically a parasite of rodents but feeds freely on a variety of animals as well as on man. Transmission of relapsing fever in the United States takes place by the bite of the male and female ticks in any stage of development, and not by means of the coxal fluid as with *O. moubata* in Africa.

Biology and Classification. The habits and life histories of the Argasidae are strikingly different from those of the Ixodidae. The "soft" ticks are intermittent feeders, being comparable in this habit to the bedbug. They feed primarily at night and do not tend to remain on their host. These ticks live and breed in the nest or lairs of the hosts to which they have relatively ready access. Both nymphs and adults are rapid feeders and are capable of withstanding long periods of starvation. The female argasid, in contrast to the ixodid, deposits comparatively few eggs (100 to 200) in each of several batches.

Pavlovskii and Skruinnik (1945) demonstrated that *Ornithodoros tholozani* could transmit the spirochetes of relapsing fever, with which they were naturally infected, after a period of 10 years. Previous investigations have shown that this species can live up to 25 years and is capable of withstanding starvation for periods up to 7½ years. These facts alone, coupled with the ability of these various species to feed on a wide variety of hosts, and their tendency to remain in a restricted area, ensure the maintenance in nature of endemic foci of relapsing fever.

Cooley has recently erected four genera in this family: *Argas, Otobius, Antricola,* and *Ornithodoros.*

Key to Genera of Argasidae

1. Adults and nymphs with a definite sutural line separating the dorsal and ventral surfaces; body flattened, oval or rounded with a distinct flattened margin ..... *Argas*
   Adult and nymph without above characters 2
2. Hypostome vestigial in adults, well developed in nymphs; body integument of nymphs covered with spines ..... *Otobius*
   Hypostome never vestigial in adults or nymphs; body integument often tuberculated but lacking distinct spines 3
3. Hypostome broad at the base and scooplike (parasites of bats) ..... *Antricola*
   Hypostome variously formed but never scooplike (parasites of bats and other animals) ..... *Ornithodoros*

Control. Caves should be avoided as sleeping quarters and cabins should be rodent-proofed in areas where tick-borne relapsing fever occurs. Floors should be cleanly swept and hammocks, cots, and beds raised from the floor and away from the walls. Old camp sites and resting places should be avoided as far as possible while traveling.

CHAPTER 27

The Insects

The class Insecta (Hexapoda) is the largest and most important group in the phylum Arthropoda and probably represents the dominant form of animal life upon the face of the earth. The insects comprise about 70 per cent of the known species of animals; over 600,000 having already been described.

As might be inferred from their enormous representation among living things, insects are probably more beneficial and, at the same time, are more injurious to man than any other group of animals. From a medical standpoint, their primary importance is due to the fact that they are either directly injurious to man or are the vectors of some of the most dangerous pathogenic organisms.

Classification and Morphology. Most adult insects may be readily distinguished from other arthropods by the separation of the body into three distinct regions—head, thorax, and abdomen. The head bears eyes, mouth parts, and a pair of antennae. One pair of legs is found on each of the three segments of the thorax. Wings, when present, occur on the second (meso) and third (meta) thoracic segments; when there is but a single pair they are attached to the mesothorax. The abdomen is made up of a series of segments, varying in number and bearing no appendages except the terminalia; each abdominal segment consists of a dorsal plate (tergite) and a ventral plate (sternite) connected by membranes to permit expansion and contraction of the body. Most insects undergo marked changes in form during the course of their development from egg to adult, a feature of major importance when control measures are considered. Two types of metamorphosis are recognized; these depend upon the degree of change which takes place during growth to maturity. Lice are common examples of insects with an *incomplete* metamorphosis; here immature stages resemble the adults very closely except for size. The bedbug and cockroach have an incomplete metamorphosis, but the young, or nymphs, exhibit a greater change in growth of the body, development of wings, and genital appendages. Control measures do not vary greatly for the different stages of such insects, since the environment, anatomy, and food habits of both young and adult are very much alike.

Other insects, such as flies, fleas, and mosquitoes, undergo a *complete* change in appearance, and usually a different ecologic habitat is evident in their development from egg to adult. In many of these insects the mouth parts of the immature and adult forms are of totally different types, involving entirely distinct feeding habits; thus, control measures will differ greatly, depending upon the stage to be destroyed.

Species from nine orders are known to affect the health of man to a greater or lesser degree, and these orders will be discussed in detail:

Order Orthoptera (cockroaches)
Order Coleoptera (beetles)
Order Lepidoptera (butterflies)
Order Hymenoptera (bees, ants)
Order Ephemeroptera (mayflies)
Order Hemiptera (true bugs)
Order Anoplura (lice)
Order Siphonaptera (fleas)
Order Diptera (flies)

Cockroaches (Order Orthoptera)

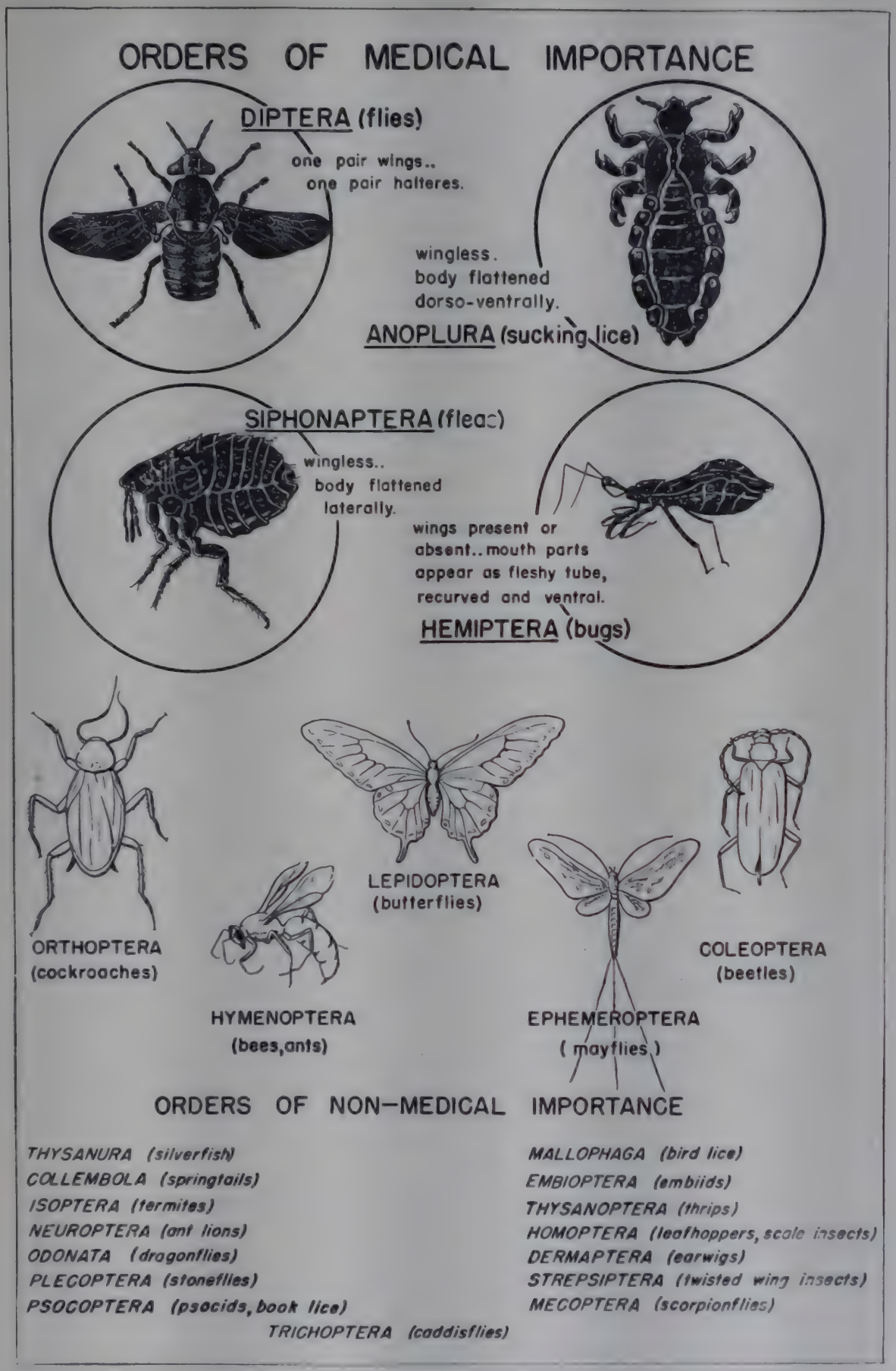
Cockroaches are omnivorous and have been under suspicion for many years as mechanical carriers of certain human diseases. They have filthy habits, feeding on human excreta as well as on nearly all the foods consumed by man. The causative organisms of tuberculosis, leprosy, amebiasis, bacillary dysentery, and cholera have been found to pass unharmed through their intestinal tracts. Contamination of food may be accomplished either by contact or by fecal deposits. The cockroach may also serve as intermediate host of certain rare parasitic worms.

Biology and Morphology. Cockroaches are characterized by having a disclike pronotum, a flattened body, long filamentous antennae, and strong legs adapted for running. The adults usually have two pairs of wings, the outer pair appearing leathery. The eggs are assembled within the body of the female and are enclosed in a hard capsule known as an *oötheca*. This structure is carried partly extruding from the abdomen of the female. After several weeks it is deposited in some dark corner or crevice. On hatching, the young are small, white, and very soft. The metamorphosis is incomplete, for the young resemble the adults except for size, presence of wings, and development of sexual organs. Cockroaches are nocturnal in habit and conceal themselves for protection from light and natural enemies.

At least five species have become domesticated and are generally distributed as common household pests.

Key to Some Common Adult Cockroaches

1. Fore pair of wings well-developed, extending to or beyond the tip of abdomen . 2
   Fore pair of wings represented by small oval pads in the female, extending one-half the length of the abdomen in the male; color black or brown (oriental cockroach) ..... *Blatta orientalis*
2. Body about 12 mm. in length 3
   Body 25 mm. or more in length 4
3. Pronotum with two dark, longitudinal stripes; wings straw-colored (Croton bug, German cockroach, water bug) *Blattella germanica*
   Pronotum without longitudinal dark stripes; wings marked with two light-yellow cross bands (tropical cockroach) *Supella supellectilium*



Orders of the class Insecta.

4. Wings uniformly reddish-brown, with a heavy yellow line on the outer edge of the basal half (Australian cockroach) *Periplaneta australasiae*
Wings uniformly reddish-brown, without such markings (American cockroach) ..... *Periplaneta americana*

Control. Thorough cleanliness and protection of food supplies are important measures in roach control. In loosely constructed buildings where a mild climate prevails, reinfestation is common and control must be periodic. *Sodium fluoride,* although poisonous to man, is the best-known roach remedy. It should be thoroughly dusted or blown into hiding places where roaches run most frequently. This agent acts as a stomach poison, being ingested when the insect cleans its legs and antennae. Application should be repeated at intervals of one to two weeks, or until the roaches disappear. Borax powder and phosphorous pastes may also be used, although they are not so effective as sodium fluoride.

Kitchens and dining rooms may be treated with DDT. The 5 per cent emulsion, the 5 per cent solution in kerosene, or the 10 per cent dust may be sprayed or dusted into the runways and likely hiding places of young and adults. Pyrethrum sprayed about the room acts primarily as a stupefying agent on the cockroaches; after its use the insects should be swept up and destroyed before they crawl away for recovery.

Beetles (Order Coleoptera)

Beetles feeding on fecal material may serve as intermediate hosts of certain helminth parasites of man and animals—*Gongylonema pulchrum, Moniliformis moniliformis, Macracanthorhynchus hirudinaceus,* and *Hymenolepis diminuta.* The blister beetles (family Meloidae) carry a toxic substance diffusely scattered throughout the body which may blister the skin of man through simple contact with the living insect or from touching the crushed beetle. Cantharidin, a local irritant occasionally used in medicine, is prepared from certain species. Swarts and Wanamaker (1946) reported several cases of bullous lesions on the legs and necks of soldiers produced as a result of contact with a small common species of the genus *Epicauta.* Canthariasis of the digestive tract, urinary system and skin have been reported. Sharpe (1947) reported an unusual case of intestinal canthariasis produced by the larvae of the beetle *Ptinus tectus.* This species is a common pest of dry food products and the eggs were ingested with the food, hatching in the alkaline portion of the gut.

Butterflies, Moths (Order Lepidoptera)

Caterpillars of certain adults of Lepidoptera possess special hairs with poison-gland cells located at their bases. The fluids contained in these cells irritate the skin and produce an extensive rash and dermatitis upon contact. Hairs from the larvae of the browntail moth cause "browntail" rash; other well-known larvae with nettling hairs are those of the flannel, io, and buck moths and the saddleback caterpillar. Berkowitz (1945) reported numerous cases of urticaria among troops in New Guinea. *Ochrogaster contraria* is the common caterpillar in Australia, the hairs of which have been reported by Flecker and McSweeny (1944) to pro-

duce an irritative dermatitis. *Megalopyge opercularis,* popularly known as the puss caterpillar, is a common species occurring in southern United States.

Bees, Ants (Order Hymenoptera)

The venom of bees is injected through the sting located at the end of the abdomen. If the stinging apparatus has been left in the wound it should be removed to prevent the discharge of additional venom from the poison sac. As a rule, the effects of a sting are entirely local, but general symptoms—fever, dizziness, dyspnea, and urticarial lesions—have been reported. The introduction of venom into the conjunctiva may give rise to more serious reactions, the tissues becoming greatly inflamed and edematous.

It has been recently suggested that bees are not uncommonly associated with allergic reactions in persons sensitive to pollen or other substances which bees may bring into close proximity with human beings. The term "bee allergy" has been applied to this condition.

Certain species of wasps have been reported to act as mechanical carriers of the eggs of *Ascaris, Ancylostoma,* and *Trichuris* through the agency of their wings, legs, and body.

The bites and stings of ants are of little significance in temperate regions, but the large, formidable species in the Tropics may not only cause local irritation but may produce general symptoms of nervous system involvement. The large ants of Central America and northern South America, particularly the "tucandeira" (*Paraponera clavata*), are especially feared by the natives because of very painful lesions which follow their stings. In parts of India and Africa, tropical foraging ants of the genus *Monomorium* produce lesions by the bites of their mandibles.

Mayflies, Lakeflies (Order Ephemeroptera)

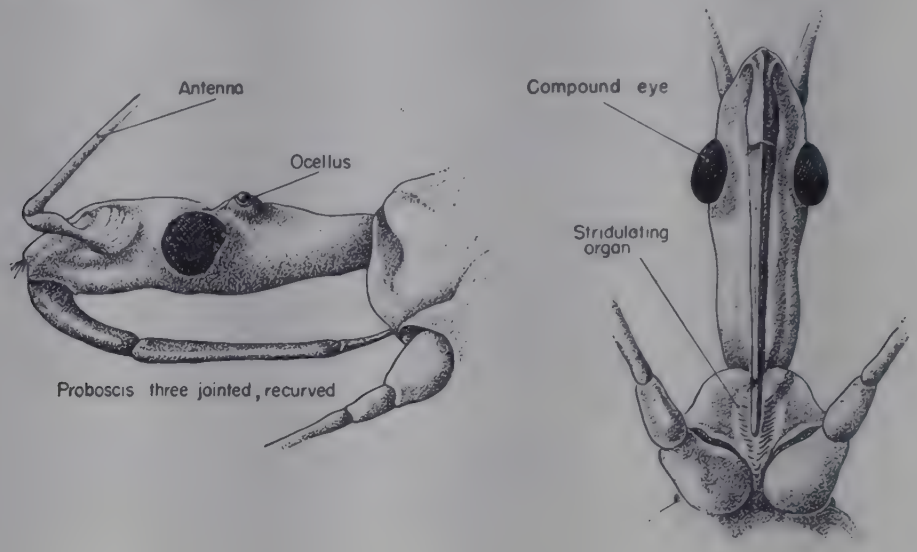
Members of this order are of medical interest in certain areas of the United States because of the allergic conditions which they produce. Along the shores of Lake Erie a common species of *Hexagenia* occurs in midsummer in such numbers that the cast skins are to be found everywhere. Persons breathing fragments of these skins may become highly sensitized and suffer severe asthmatic paroxysms.

True Bugs (Order Hemiptera)

The members of this order are characterized by the prominent beak, or proboscis, bent back under the head and thorax when in repose. It appears as a single fleshy tube arising from the lower front portion of the head. The adult bugs possess two pairs of wings; these are rudimentary in some genera. Metamorphosis is incomplete. The majority of species feed on a wide variety of plants and many are adapted for aquatic life. Only a few of the bugs are blood-suckers, and these occur in two families:

Family Cimicidae (bedbugs)
Family Reduviidae (conenose bugs)

Bedbugs (Family Cimicidae). Bedbugs have long been suspected to be vectors of human diseases, but to date there is no conclusive evidence that they are important in the transmission of any disease in nature. The bite of a bedbug is generally not felt immediately, but the salivary secretions may cause itching, burning, and swelling; in some cases, secondary infections are started by scratching the site of the bites.



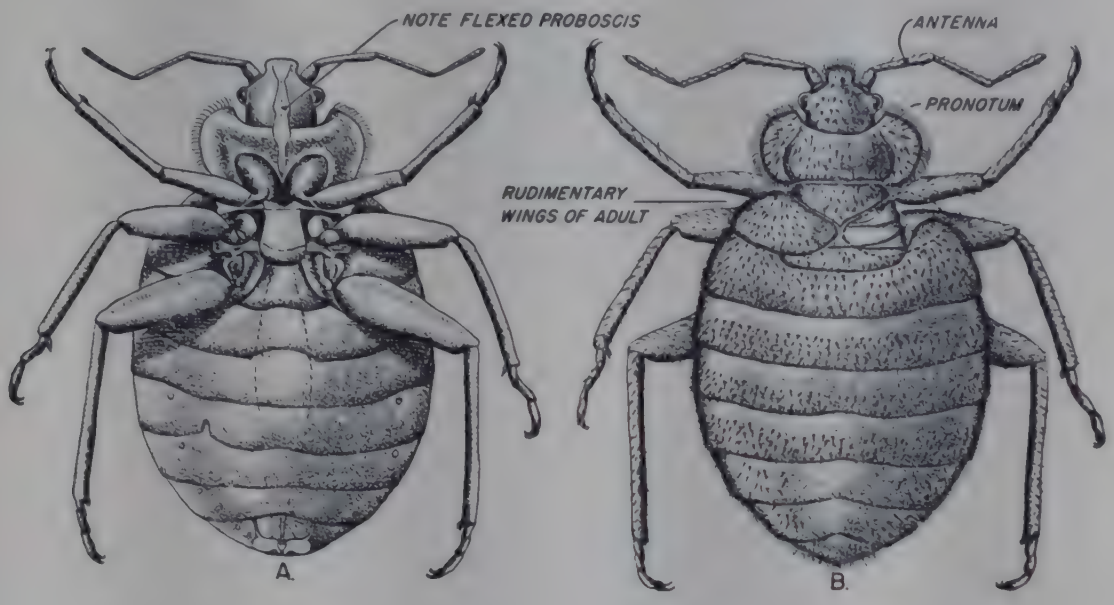
Head and proboscis of a bloodsucking hemipteron. Note stridulating organ on the prosternum, characteristic of the Reduviidae.

Biology and Morphology. Members of this family have a flattened body, are usually mahogany in color, and possess a four-segmented proboscis (three apparent). The antennae are four-jointed. Wings are rudimentary and appear only as mere pads on the body. The adult is about 5 mm. long and 3 mm. broad and is especially adapted by its flattened body for entering cracks and crevices to hide during the day. The rather broad head is set deeply into the prothorax between the large lobes formed by the pronotum. The female deposits about 50 eggs at a time and in about 10 days the young emerge. Four generations may develop in a year; the average period of life is probably three to six months. *Cimex lectularius* is the cosmopolitan bedbug; *C. hemipterus* is the common species found in the Tropics, especially in Asia.

Control. DDT is the most desirable of all available insecticides for the control of bedbugs. A 5 per cent kerosene spray, or the 10 per cent powder applied to mattresses and other likely hiding places will give a residual effect lasting for six months or more. Fumigation with hydrocyanic acid gas is effective but requires trained personnel. One pound of sodium cyanide is usually sufficient for 1000 cubic feet of air space.

Conenose Bugs, Assassin Bugs (Family Reduviidae). Most of the species in this family are predaceous, feeding on other insects; some species are plant feeders. A few are mainly dependent on wild animals for a blood meal, and certain species have become adapted to human habitations and are of medical importance as the intermediate hosts of *Trypanosoma cruzi* which causes Chagas' disease. The common method of transmission of the disease is through the agency of the

reduviid bugs, especially *Panstrongylus megistus.* In northern Argentina and Uruguay the more common transmitting species appears to be the "unchuca" (*Triatoma infestans*). However, the parasite is capable of developing in a number of other species of *Triatoma* and allied genera, as *Rhodnius, Eratyrus,* and *Eutriatoma.* Some 26 species of the blood-sucking reduviids (sometimes treated as



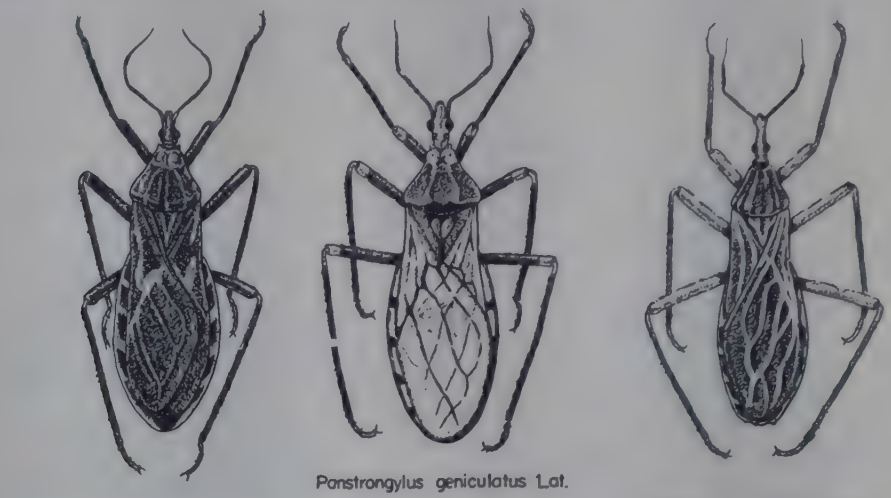
Cimex lectularius, the bedbug: (A) Ventral view. (B) Dorsal view.

a separate family Triatomidae) have so far been found naturally infected with *T. cruzi* and are capable of transmitting infection (Torrealba, 1946). In California, Kofoid found *Triatoma protracta* to be infected, while Reichenow (1934), in Guatemala, found the transmitting agent to be *T. dimidiata,* and Clarke and Dunn (1932), in Panama, have shown that *Panstrongylus geniculatus* is the usual transmitting arthropod. *Eratyrus cuspidatus* was also found naturally infected. Shuck (1945) reports *Triatoma longipes* and *T. rubida* as being naturally infected in Arizona. While bugs infected with *T. cruzi* have been recovered in the United States, no naturally infected human cases have been reported. The reduviid deposits infective feces as it feeds and these are rubbed or scratched into the excoriated skin at the site of the bite. Many species are said to produce painless bites; others inflict painful bites when disturbed. *Triatoma sanguisuga* has recently been reported by Kelselman and Grundman (1940) in Kansas as naturally infected with the western strain of equine encephalomyelitis.

Biology and Morphology. Members of the family Reduviidae are characterized by the three-segmented proboscis which arises from the anterior portion of an elongate head. When viewed laterally, it appears as a shallow loop with the tip resting just forward and between the prothoracic legs in a prosternal furrow. Important genera can be recognized by the position and insertion of the antennae.

The eggs are deposited in dusty corners and crevices of houses or in nests and burrows of the hosts; nests of the wood rat (*Neotoma*) are favorite places to collect many species. Reduviids tend to be nocturnal in feeding habits; both sexes bite man.

Control. No effective control measures are known for these bugs, and eradication is very difficult because of their habits. Mosquito nets and screened quarters may offer some protection against adults and the larger nymphs.



Three medically important reduviids.

Sucking Lice (Order Anoplura)

The body louse (and probably the head louse) is known as the vector of epidemic typhus and trench fever. It has been shown to transmit relapsing fever in Europe, India, China, northern Africa, and North America. Transmission is accomplished when the crushed louse or its feces is scratched into the bite or excoriated skin; the bite alone is not sufficient. A characteristic scarring or bronzing of the skin (vagabond's disease) often occurs, due to toxic substances secreted during feeding. This melanoderma is especially marked where large numbers of lice have fed. Crab lice are not generally incriminated as vectors of disease although Chung (1944) points out that the crab-louse may act as a transmitter of typhus under certain conditions in which case they serve merely as reservoirs of the infected blood.



Pediculus humanus var. *corporis,* the body louse.

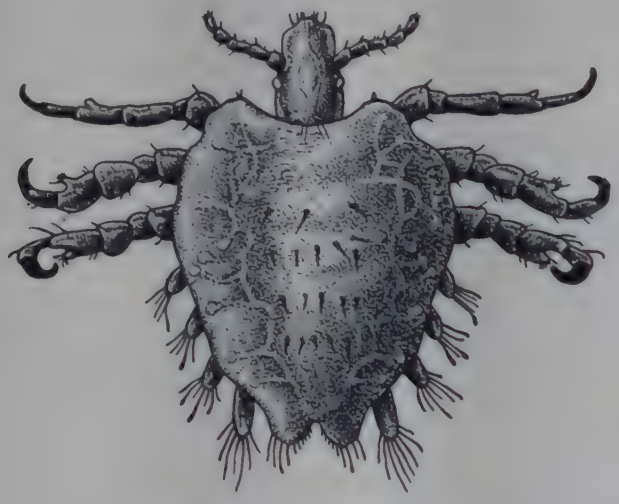
Sucking lice are small, dorsoventrally flattened, wingless insects which undergo an incomplete metamorphosis. Their legs possess one-jointed claws which are well adapted for clinging to hairs. The insects included in this order are exclusively blood-sucking ectoparasites. About 200 species are known, and of these two infest man.

Body Louse (Pediculus Humanus var. Corporis). Eggs of the body louse are laid among the folds of the clothing at the rate of 8 to 12 daily; as many as 300 eggs are laid by one female. Young and adults live among the folds and seams of clothing, being rarely found on the skin except when feeding or when extremely abundant. The preferred location for feeding is in the region of the neck. Blood is sought frequently through-

out the day and night, and deprivation of food causes death of the adult in about 10 days and of the newly hatched nymph in about two days.

Head Louse (Pediculus Humanus var. Capitis). The eggs of the head louse are deposited on the hairs of the head; the young mature in about 10 days. This variety of *P. humanus* differs but little in structure and in no essential manner in its life history from *P. humanus* var. *corporis;* typical forms of the head louse are usually smaller and darker, with the antennae somewhat thicker.

Crab Louse (Phthirus Pubis). This species is popularly known as the crab or pubic louse. The first pair of legs is slender and is terminated by a straight claw. Legs of the second and third pairs are supplied with large, hooklike claws. The crab lice usually infest the pubic and perineal regions; in heavy infestations, they may be found on the eyebrows or hair of the armpits. The eggs hatch in about seven days, and the life cycle extends between 20 and 25 days.



Phthirus pubis, the crab louse.

Control of Sucking Lice. The new synthetic compound, DDT, is more highly effective and longer lasting than any other previously known insecticide and will no doubt be used exclusively in the control of the vectors of epidemic typhus and louse-borne relapsing fever; 10 per cent dust applied to the inner surface of clothing worn next to the skin will give protection until the clothes are washed. Methyl bromide, effective against nits, may be used as a fumigant for clothing and bedding. Steam is also efficient, causing but little shrinkage of woolen goods; an improvised apparatus for steam sterilization is easily assembled. Permanent delousing stations with pressure sterilizers are used, but they are being supplanted by DDT. Clothing and hair should be inspected regularly. Frequent bathing and changes of clothing will assist in control. Wheeler (1946) discusses the remarkable success achieved in Naples and southern Italy in 1943–44 as a result of mass dusting operations for the control of louse-borne typhus.

Fleas (Order Siphonaptera)

Fleas affect the health of man by acting as vectors of plague and endemic typhus (murine typhus) and as intermediate hosts of the dog tapeworm, *Dipylidium*

caninum. The bites of fleas may cause a severe dermatitis in susceptible individuals; certain species (chigoes or sand fleas) invade the epidermal tissues of man, producing lesions that are likely to become secondarily infected. Although various animals usually have species of fleas peculiar to them, these insects are not host specific and when hungry they will readily attack any warm-blooded animal; such ready transfer greatly increases their potentialities as transmitters of disease to man.

The species primarily responsible for the transmission of plague is *Xenopsylla cheopis,* the Indian rat flea, which is considered to be the most common vector of murine typhus. *Nosopsyllus fasciatus* is the common flea of *Rattus norvegicus* in Europe and the United States and, although not prone to attack man readily, it is a capable vector of plague and typhus among rats and mice. *Diamanus montanus* and *Hoplopsyllus anomalus* are known vectors of plague among wild rodents in California and Oregon. The cosmopolitan human flea of Europe and the United States is *Pulex irritans*. *Ctenocephalides canis* and *C. felis,* the dog and cat fleas respectively, are commonly found infesting houses in the United States, but they are not important natural vectors of disease.

Tunga penetrans, the jigger, chigoe, nigua, or sand flea, is of great importance in a number of tropic countries. The male and virgin female are relatively unimportant, since they do not penetrate the skin. The impregnated female bores its way into the skin about the toes, the soles of the feet, or the fingernails and develops enormously, increasing from 1 mm. in length to nearly the size of a small pea. The "sticktight" flea, *Echidnophaga gallinacea,* closely resembles *Tunga penetrans*. It is a serious pest of poultry in southern United States and in tropic and subtropic countries and occasionally attacks man, dogs, and cats.

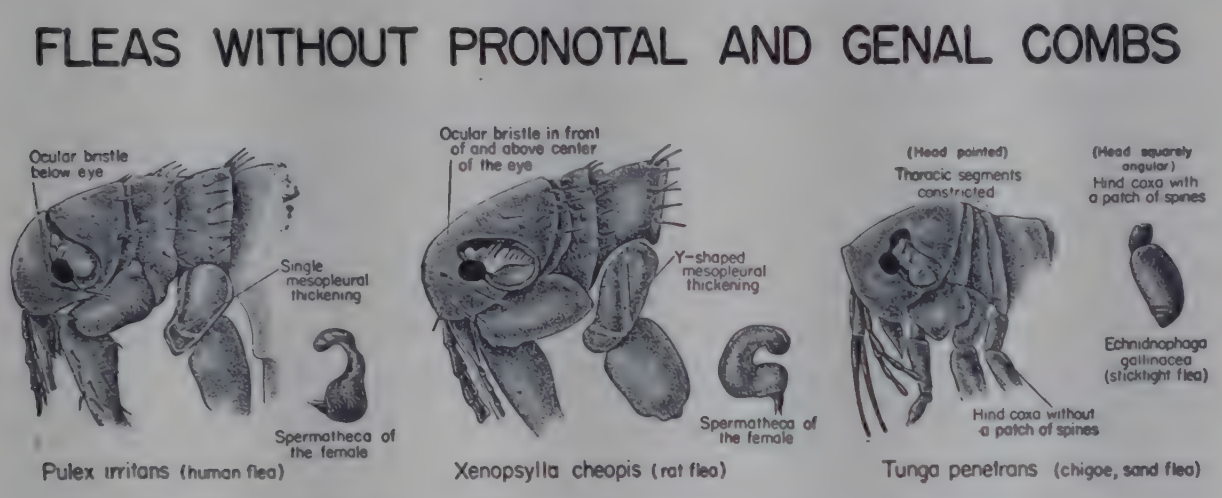
Biology and Morphology. Fleas are small, wingless, laterally compressed insects which live as ectoparasites on mammals and birds. The adults are medium to dark brown in color and have mouth parts adapted for piercing the skin. Several families are recognized with difficulty, although such characters as the number of rows of spines on the abdomen, the presence or absence of eyes, the presence or absence of a genal or pronotal comb, and the number of teeth in each, the shape of the spermatheca, and the structure of the mesopleuron are all valuable aids in making tentative identification of the commonly occurring fleas. The oval eggs, unlike those of many ectoparasites, are deposited on the floor or in the nests of their hosts. Metamorphosis is complete. The larvae feed on available organic debris and are found with the pupae in the nests or on the floor, rarely on the host. The length of the life cycle varies with species and environmental conditions. Fleas previously fed have been known to survive for as long as 966 days; unfed, they may survive for many months.

Control. Strict cleanliness in private houses or public buildings prevents fleas from breeding in them; dusty cracks and unswept carpets furnish excellent habitats for many species. Infested houses may be rid of fleas, except for the eggs, by sprinkling the floors with naphthalene and sealing the rooms for several days, or by scattering sodium fluoride or pyrethrum powder on the floors. Fleas on rats can

best be controlled by eradication of the rodent host through fumigation. Ten per cent DDT powder may be applied to the beds of dogs and cats and to runways and holes used by rats. It may be used beneath rugs, on floors, or soil that is visited by flea-infested animals; one-half pound will treat 1000 square feet. The 5 per cent DDT-kerosene spray is most effective.

Rodent Control. It has been noted that a high death rate among rodents as a result of a plague epizoötic often precedes an outbreak of human plague. When rats or mice are reduced in numbers, there is more likelihood that the rodent fleas will seek the body of man for food; consequently, it is well to employ measures that will destroy both rodents and fleas simultaneously.

Exhaust gases from automobiles or other gasoline engines (carbon monoxide), calcium cyanide, or carbon bisulfide may be used in destroying rats in their



Identification characters of some medically important fleas occurring in the United States.

burrows. Calcium cyanide, which gives off hydrocyanic acid gas, is a dust and may be used by placing 1 tablespoon down each burrow and closing the entrance immediately. Large-scale fumigation, as carried out for ships, should be done only by experienced personnel. Stewart and Mackie (1938) have found that liquid methyl bromide is very effective in destroying rodents in their burrows. In areas where fumigation is not feasible, active trapping with spring traps and poisoning through the use of the agents listed below may be recommended:

Kind of Poison	*Amount*	*Bait*
Barium carbonate	4.00 oz.	1 lb.
Red squill	16.00 oz.	9 lbs.
Thallium sulfate	0.25 oz. (½ level teasp.)	1 lb.
Strychnine alkaloid	0.1 oz. (2 level teasp.)	1 lb.
Arsenic trioxide	0.5 oz. (3 level teasp.)	1 lb.

Bread has proved to be a very successful bait when used with any of the above poisons. The selected poison is mixed with the broken or ground bread in the proportion mentioned and water added until the mixture will form a ball when compressed in the hand. Portions may be wrapped in wax paper to form "torpedoes" and placed in areas known to be frequented by rats. Coconuts, bananas, sweet potatoes, meat, melons, or peanut butter may also be used as fresh baits.

In controlling the ship-shore-ship rodent movements, rat guards or shields should be maintained on all connecting lines. Light clusters should illuminate bow and stern lines at night; all landing stages, gangways, and cargo nets should be removed from the wharves and between ships during darkness, in so far as is possible.

Mosquitoes, Flies, Gnats (Order Diptera)

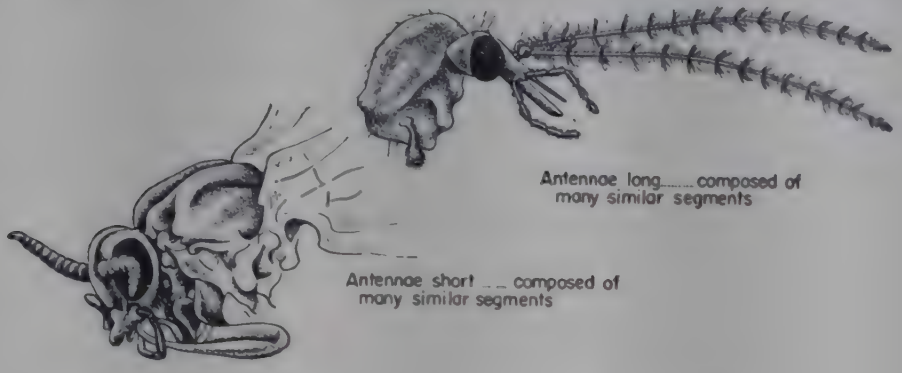
The order Diptera comprises one of the largest single groups of insects, containing over 75,000 species. Structurally, these invertebrate animals are the most highly specialized of the class Insecta. All forms that are properly termed "flies" belong to this group. However, the word "fly" forms a part of the compound name of certain insects, such as butterfly, mayfly, or dragonfly, which are not members of the order Diptera. Nearly all species of the Diptera have sucking mouth parts, and a few possess structures capable of piercing the skin of man. These skin-piercing forms are the most important disease vectors and must be regarded as potential transmitters of any pathogenic microörganism. With few exceptions, flies with nonpiercing mouth parts cannot be held responsible for introducing infection into the body except through previously injured surfaces; however, they may be highly efficient in the mechanical transfer of parasites.

Classification. The characteristic feature of the Diptera is the presence of only one pair of functional wings; the second pair is reduced to knoblike structures known as *halteres*. With few exceptions, flies undergo a complete metamorphosis: egg, several larval stages, pupa, and adult.

Because of the enormous size of this order and the additions and corrections

which are constantly being made, classification and identification have always been difficult and unsatisfactory to the laboratory worker. A practical method of classification is used at the U. S. Naval Medical School where, for convenience, members of the order Diptera are placed in one of three suborders—*Nematocera, Brachycera,* or *Athericera*—according to the shape and structure of the antennae. This classification is based upon visible anatomic features; since adult specimens are generally submitted for identification, the antennae provide adequate clues to the medically important groups.

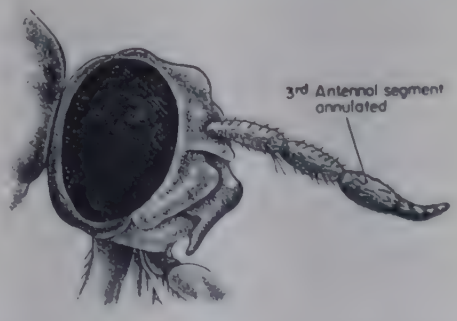
Suborder Nematocera (Orthorrhapha, in part)—Mosquitoes, Many Mosquito-like Gnats, Black Flies, Midges, Sand Flies. The antennae of adults are



Antennal characters of the suborder Nematocera.

composed of 6 to 20 similar segments, the two basal ones usually differentiated from the rest. The palpi are nearly always filiform and four- or five-jointed. The larvae have a well-developed head with chewing mouth parts. Disease vectors belonging to this suborder have *mouth parts which are capable of piercing the skin of man. Only the females bite;* the males obtain their nourishment from plant juices. Transmission of disease usually *requires a lapse of time, during which the vector is noninfective.*

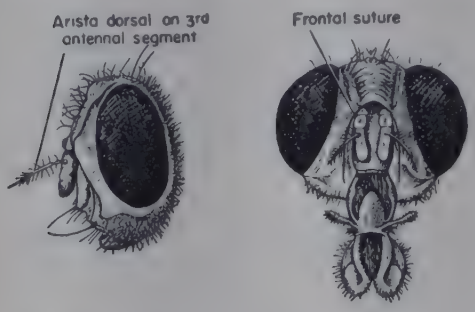
Suborder Brachycera (Orthorrhapha, concluded)—Horse Fly, Deer Fly, etc. The antennae are variable, generally the two basal segments are differentiated from the third which is frequently subdivided into a number of false segments or annulations; arista, when present, is rarely dorsal, usually terminal; in living specimens, the antennae extend in front of the head, bayonet fashion. The larvae have an indistinct head and retractile, vertically biting mouth parts (mandibles). The medically important members of this suborder have *piercing mouth parts. The females alone can bite;* the males feed on plant juices. Disease transmission may be *direct or may require a lapse of time during which the insect vector is noninfective.*



Antennal characters of the suborder Brachycera.

Suborder Athericera (Cyclorrhapha)—Tsetse Fly, House Fly, Green Bottle Fly, etc. The antennae are three-segmented. The spinelike *arista* is subject to wide variation in regard to position and structure, but is usually located dorsally

on the base of the third segment. A frontal lunule is present in nearly all medically important forms, and a frontal suture is usually present. The larvae are without a distinct head and the mouth parts are reduced to simple hooks. The pupae are enclosed in the last larval skin to form a white or yellowish to mahogany-brown puparium. The majority of disease vectors, in both sexes, have *mouth parts which are incapable of penetrating the skin.* Transmission is *typically mechanical, requiring a prompt feeding on the second host for infection to take place.* Two main factors are involved in this type of transmission: (1) *contact with a contaminative source, such as an open sore, feces, garbage, or sputum and* (2) *subsequent contact with the broken skin, conjunctivae, food, or drink.* A few genera in this suborder have *piercing mouth parts; both the males and females bite* (*Glossina, Stomoxys,* etc.). Disease organisms undergo a biologic transformation in the medically important forms.



Antennal characters of the suborder Athericera.

Mosquitoes, Mosquito-like Gnats, Sand Flies, Black Flies, Midges (Suborder Nematocera)

This group is the most primitive of the three; it includes species which are important in the transmission of malaria, dengue, sand-fly fever, and many other diseases. Four families are recognized to be of medical importance:

Family Psychodidae (sand flies, moth flies)
Family Ceratopogonidae (no-see-ums, punkies, biting midges, sand flies)
Family Simuliidae (buffalo gnats, black flies, turkey gnats)
Family Culicidae (mosquitoes, mosquito-like gnats)

Sand Flies, Moth Flies (Family Psychodidae). Sand flies are the vectors of visceral and cutaneous leishmaniases. The species incriminated for *Leishmania donovani* (kala-azar) are *Phlebotomus argentipes* in India; *P. chinensis* and *P. sergenti* in China; and *P. major, P. sergenti,* and possibly *P. papatasii* in the Mediterranean region. *Phlebotomus papatasii* and *P. sergenti* are believed to transmit *L. tropica* (oriental sore) in the Old World, and *P. intermedius* to carry *L. brasiliensis* (espundia) in South America. It is believed that both visceral and cutaneous leishmaniases are transmitted by the bite of the insect; crushing the fly may be important in the transmission of the cutaneous type.

Phlebotomus papatasii is a proved vector of sand-fly or pappataci fever throughout the Mediterranean region, the Balkans and Asia eastward to India. *Phlebotomus verrucarum* and *P. noguchii* are transmitters of *Bartonella bacilliformis,* the cause of verruga peruana in Peru; the distribution of the disease is restricted to the canyons along the western slope of the Andes where those species occur.

MacPherson (1941) reports that men of the Australian forces suffered severely in North Palestine from a disease referred to as "harara" and describes it as an

allergic reaction due to the bites of *Phlebotomus* at the height of sensitization. The exposed skin becomes covered with hard wheals up to 1 cm. in diameter. These may subside or may be replaced by blisters which may become infected; in some cases the regional lymph nodes become enlarged and tender.

Biology and Classification. The more common members of this family belong to the genus *Psychoda* and are harmless, nonbiting flies. They appear in large numbers in and around breeding areas, such as sewage disposal plants and cesspools, and not uncommonly about wash basins. The adults are small and mothlike owing to their dense hairy covering. The wings rest in a rooflike manner over the body. Members of the closely related genus *Phlebotomus* are blood-suckers and produce painful bites. Like members of the genus *Psychoda,* the body and wings are covered with hairs. The antennae are long and hairy and consist of about 16 segments. The palpi are four-jointed and are longer than the head. Unlike *Psychoda,* the wings are raised over the abdomen at an angle of 45 degrees when the adult is at rest.

Sand flies (*Phlebotomus*) hide in damp places during the day. Only the females suck blood, emerging primarily at night to feed. They fly only short distances and rarely rise more than a few feet above ground. In open country where breeding places are associated with widely scattered rodent burrows, Hertig (1945) reported flight ranges up to 1500 yards as not uncommon. A few days after feeding the females deposit ova (40 to 60 per insect), by preference in crevices of damp, shaded rocks, stone fences, ruins of buildings, or caves within 100 or 200 feet of their feeding places. Occasionally, they may feed a second time and deposit a second batch of ova, but their life span is short—7 to 14 days. The entire life cycle covers one to two months.



Adult *Phlebotomus,* the sand fly.

Control. Sand flies of the genus *Phlebotomus* are not easily controlled, for their breeding places are difficult to demonstrate. Repellents have been used with some degree of satisfaction in certain areas. Bites may be prevented by avoiding infested areas at night. DDT has been shown to be effective; the 5 per cent solution in kerosene should be sprayed on inner walls from floor to ceiling, reaching well into the corners, and on the framework of doors, windows, and screens, both inside and out. It should be applied at the rate of 1 to 1½ gallons per thousand square feet. Screens should be painted with the solution. Hertig (1945) reported that 5 per cent DDT in diesel oil distributed by airplane gave remarkable control of adults in the Mediterranean region.

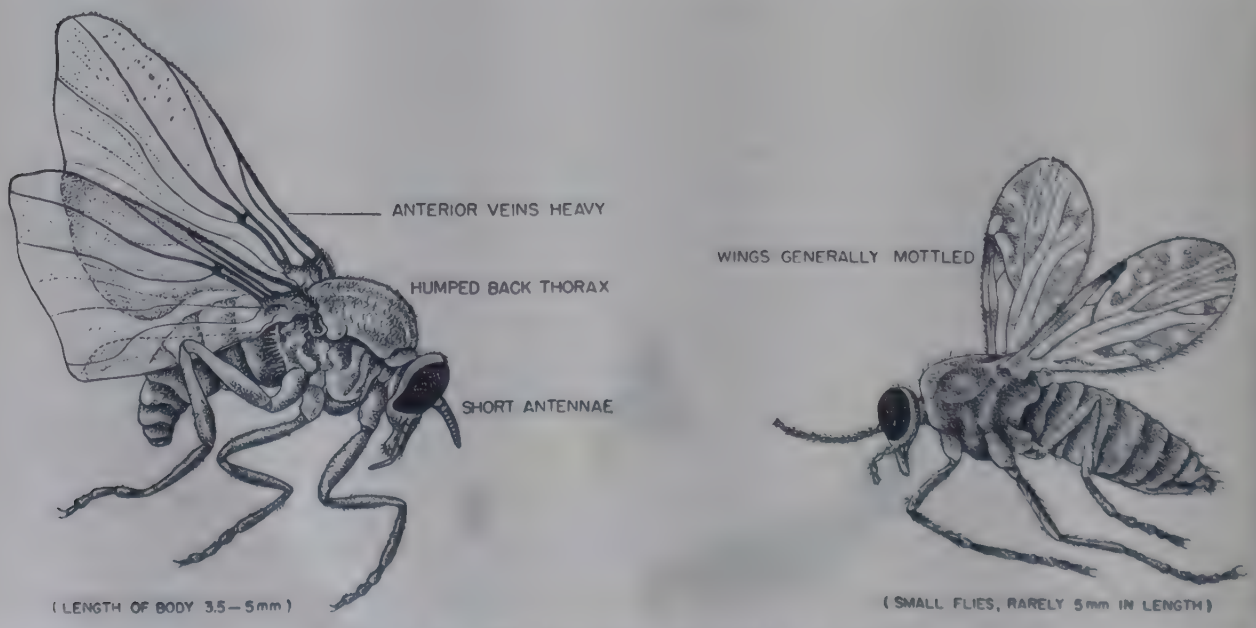
Black Flies, Buffalo Gnats (Family Simuliidae). Members of this family are extremely annoying and attack man readily in the immediate vicinity of their breeding areas, particularly during the day. In less heavily overgrown wooded areas the adults have been observed to migrate several miles. The bites are usually painless but may produce painful swellings. In northeastern parts of the United

States and in eastern Canada, black flies are the pests that probably interfere most with the pleasures of outdoor life; they are a scourge to livestock and other animals in the southern states. In addition to discomfort resulting from their bites, certain species are vectors of the filarial worm *Onchocerca volvulus,* the causative organism of onchocerciasis, or blinding filarial disease. *Simulium damnosum* and *S. neavi* are known transmitters in tropic Africa; *Eusimulium avidum, E. ochraceum,* and *E. mooseri* in Central America.

Biology and Classification. Dyar and Shannon have divided the family into four genera: *Parasimulium, Prosimulium, Eusimulium,* and *Simulium;* there are about 200 known species. The adults are rather small, hump-backed flies, usually dark in color, which probably accounts for their common names of black flies and buffalo gnats. The antennae are short, as long as or a little longer than the head. The wings are large and broad with the anterior veins thickened; the remaining veins are poorly developed.

The characteristic larvae and pupae are found attached to slightly submerged wicker-work fish traps, rocks or vegetation in rapidly flowing, clear streams. Most American species breed in swiftly flowing mountain streams and a patchy distribution is evidently due to these limited breeding habits.

Control. Black flies are rather difficult to control, and emphasis should be placed on individual protective measures. Complete clothing and head nets will limit bites over the face and body while traveling in heavily infested areas. Insect repellents will give satisfactory protection for several hours, if applied evenly over exposed surfaces. Aquatic stages may be partially eliminated by clearing rocks and logs from the streams, but such procedures are economically unfeasible for extensive control. Sudden changes in water level were shown by Wanson and Henrard (1945) to be an effective method of control of *S. damnosum* in the Belgian Congo, whereas a gradual rise or fall in water level will permit the larvae to migrate to other suitable environments. Fairchild (1945) reports success in the use



(*Left*) Adult simuliid, the black fly. (*Right*) Adult *Culicoides,* a biting midge (commonly known as "punkies," "no-see ums," or "sand flies").

of DDT emulsion in flowing streams, applied at the rate of 1 quart of DDT to 7,000,000 parts of water.

Biting Midges, Sand Flies, No-see-ums, Punkies (Family Ceratopogonidae). These flies appear chiefly in the evening and early morning and occur locally in numbers sufficient to make life almost unbearable. The bites of some species are very painful and extremely irritating, causing nodular swellings that itch persistently for several days or weeks. The common name "sand fly" is applied to members of this family as well as to the genus *Phlebotomus* in the family Psychodidae. Use of this common name may cause confusion, and a correct identification of the offending form is necessary before control measures can be instituted. Members of the genus *Culicoides* serve as the intermediate host of the filarial worm, *Acanthocheilonema perstans; C. furens* was shown by Buckley to be the intermediate host of *Mansonella ozzardi* on St. Vincent Island.

Biology and Morphology. These small, slender flies may be distinguished by the peculiar venation of the wings; the first two veins are very heavy, the others rather indistinct. Scales are absent and the wings are frequently covered with microscopic, erect hairs variously marked with iridescent areas in many species. The proboscis, like that of the simuliids, is very short. The antennae are 14-segmented. Eggs are laid in a variety of places, mainly in water or water-saturated sand and soil; larvae are commonly collected in tree holes. Throughout the Pacific Islands, large numbers of larvae and pupae have been found in water held in the numerous coral pockets above high-tide level. The slender brown pupae superficially resemble those of the mosquitoes, but unlike the latter they float almost motionless in a vertical position. The entire life cycle requires from 6 to 12 months. This family includes about 22 genera.

Control. Species of the genera *Culicoides* and *Leptoconops* have been controlled by selective residual treatment with DDT sprays or dusts. Their breeding places are usually limited and must be accurately determined before area treatment is begun. Bed nets and screens treated with DDT will poison many of these small gnats as they make their way through the meshes, but it may not kill them in time to afford complete protection from bites. Mosquito repellents are of value in protecting the hands, face, and ankles.

Mosquitoes, Mosquito-like Gnats (Family Culicidae). Mosquitoes have long been notorious as pests of man and animals, but this role is overshadowed by their importance as vectors of organisms pathogenic to man. From a medical viewpoint they are the most important of all blood-sucking diptera, being known transmitters of malaria, dengue, yellow fever, filariasis, and the various encephalitides.

Classification. Members of the family Culicidae are small, slender flies. The typical antenna is composed of 14 to 15 segments. The wings are long and narrow, and folded over the abdomen when at rest. The venation is characteristic: six longitudinal veins are nearly always present; the second, fourth, and fifth veins are forked; the third vein is unbranched and arises from the anterior cross-vein at about the middle of the wing. The wing veins possess hairs or true scales, and the

hind margin is fringed with scales or simple hairs. The immature stages are always aquatic.

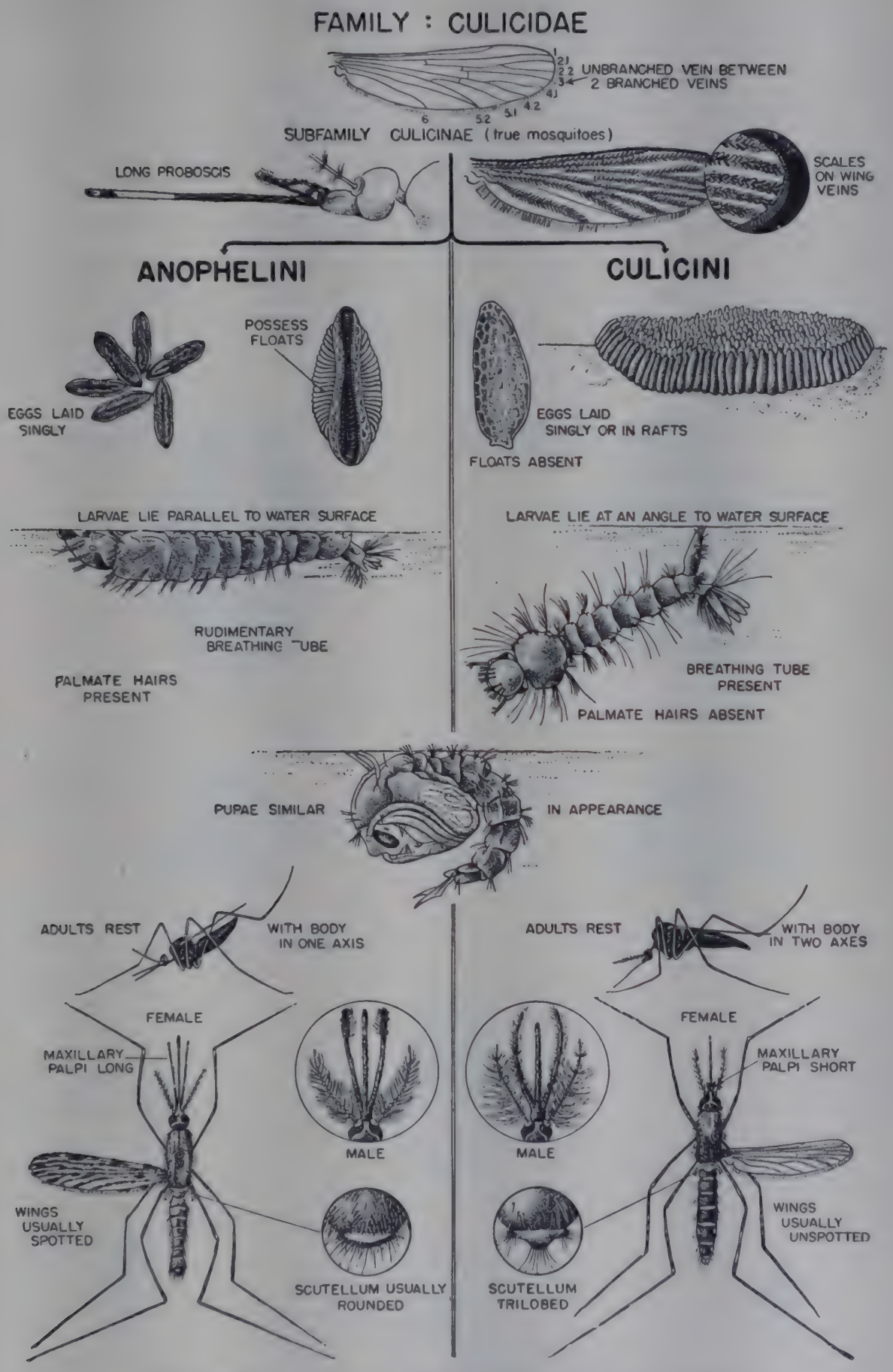
Edwards divides the family into three subfamilies: *Dixinae, Chaoborinae,* and *Culicinae.* Only the latter is of medical importance. The subfamily Culicinae contains about 1600 species, embodying the true mosquitoes, which are easily differentiated from all other mosquito-like gnats by: (1) a long proboscis (as long as the head and thorax) and (2) scales located on the wing veins, along the posterior margin of the wings, and on the legs and body. The mouth parts of the females are adapted for piercing the skin and sucking blood (although some species do not bite man or animals). Males subsist primarily on plant juices and nectars.

For practical purposes, the subfamily may be divided into two tribes, the *Anophelini* and the *Culicini.* The tribe Anophelini is further divided into three genera, of which one, *Anopheles,* is of paramount importance. (The other two genera are restricted to definite geographic areas and are unimportant as vectors of malaria: *Chagasia* to South America and *Bironella* to the Australasian region). The tribe Culicini includes the great majority of the known species of mosquitoes; it is divided into a large number of genera and subgenera, with species often separated with difficulty.

It is to be expected that within this large group of insects considerable variation would exist in the life histories of the species, and it is not possible to select any one species as typical of the subfamily. In general, all mosquitoes in their immature stages are aquatic and undergo a complete metamorphosis: egg, larva, pupa, and adult. The larvae, or "wigglers," pass through four larval stages. With the fourth molt the pupa, or "tumbler," appears. This is a nonfeeding stage during which the adult structures are organized. Usually the pupal stage is quite short, the adults emerging in 24 to 48 hours.

The various species differ greatly in rate of development, feeding habits, resting sites of adults, mode of hibernation, and choice of breeding areas. Knowledge of this "species individuality" is of great importance in disease control, for it enables the entomologist to concentrate specific control measures against the particular, offending species—*species sanitation.*

Genus Aedes. Several species of *Aedes* are particularly well known because of their ability to transmit important tropic diseases. *Aedes aegypti,* a cosmopolitan species, is the important vector of urban yellow fever and dengue; *A. albopictus* is an oriental species known to transmit dengue in the Philippines and elsewhere in the Far East. The latter has recently been introduced into the Hawaiian Islands and is responsible for the transmission of dengue in that area (Usinger, 1944). *Aedes scutellaris* is typically Australasian in distribution, although recently one species of the *"scutellaris"* group was described from the Philippines (Stone and Farner, 1945). A member of this group has been proved to be the vector of dengue in New Guinea. Mackerras (1946) demonstrated that the outbreak of "jungle" dengue fever which occurred in New Guinea was transmitted by *Aedes hebrideus.* There are epidemiologic indications that island races of *A. hebrideus* as well as other members of this *scutellaris* group vary in their ability to transmit

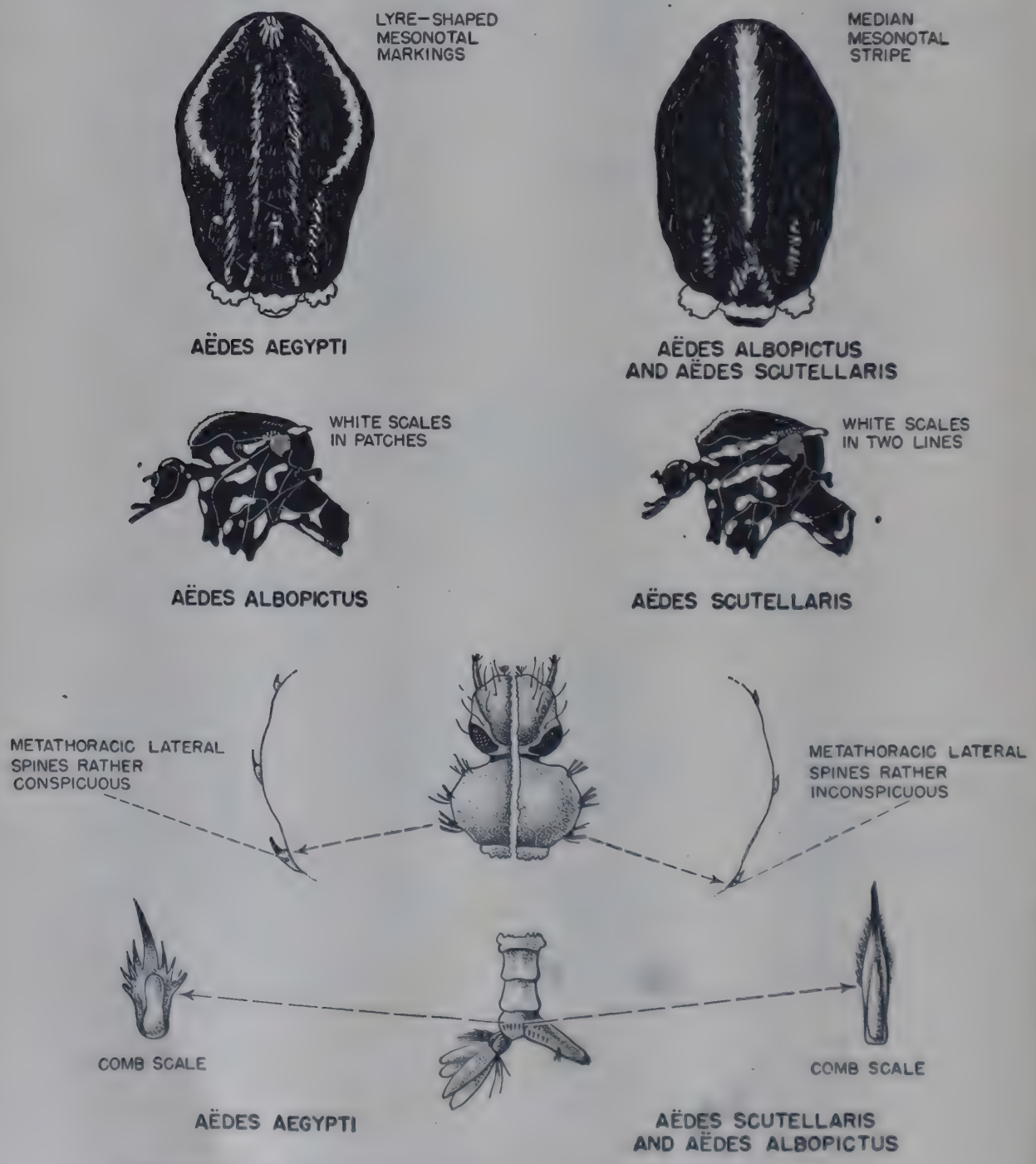


Tribal differences of the subfamily Culicinae (true mosquitoes).

dengue. *Aedes pseudoscutellaris* is the principal vector of filariasis and dengue in the Samoan-Fijian area. Several species of *Aedes* are involved in the transmission of equine encephalomyelitis.

The three important species can be separated by the thoracic markings of the adult; the mesonotum is marked by a lyre-shaped, silvery pattern in *Aedes aegypti*, but has only a median silver stripe in both *A. albopictus* and species of the *scutellaris* group. All members of the latter have conspicuous white scales arranged in two parallel longitudinal rows in addition to several scattered spots; the closely related *A. albopictus* has white scales in scattered spots but never arranged in rows.

The larvae of all three species are very similar; those of *Aedes aegypti* are characterized by the conspicuous thornlike process at the base of the lateral metathoracic hair tufts and by the prominent secondary spines on the comb scales.



Diagnostic characters for distinguishing some medically important *Aedes*.

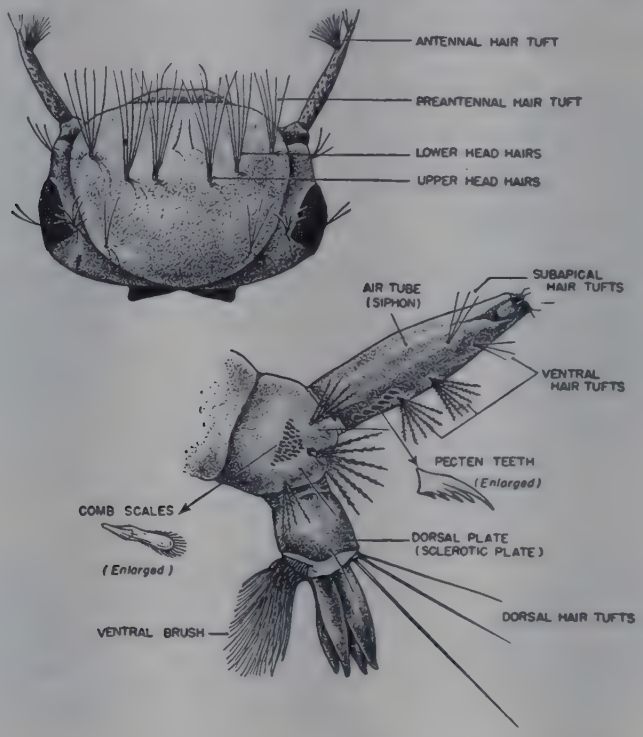
Because of the extreme variability in the larval characters of *albopictus* and *scutellaris*, separation of the various species is rather difficult. It is best to make rearings and confirm tentative identifications by examination of male genitalia and other adult characters.

The adults of most *Aedes* are avid biters, feeding throughout the day and especially in the early morning or late afternoon. Many of them are relatively weak fliers, although some salt-marsh species are known to migrate 20 to 40 miles from their breeding grounds. Bonnet and Worcester (1946) in studying the dispersal flights of *Aedes albopictus* in Hawaii found the ordinary range of flight activity to be 200 yards or less. Similar observations have been made on *A. aegypti* and members of the *scutellaris* group. Eggs are generally deposited on the ground along the margins or dried bottoms of pools, ponds, marshes, and swamps, or above the water level on the sides of various types of containers, such as tin cans, coconut husks, tree holes, cut bamboo stalks, leaf axils, or other natural cavities. These catchments may dry out completely and the eggs will remain dormant for weeks or months. When the water levels are raised the eggs become flooded and hatch almost immediately. Those of some of the northern species will not hatch during the season deposited, but remain submerged to incubate and emerge in one great brood the following spring.

GEOGRAPHIC DISTRIBUTION OF *Aedes aegypti*, *A. albopictus*, AND SPECIES OF THE SCUTELLARIS GROUP

Aedes aegypti (yellow fever and dengue)	World-wide in tropic and subtropic climates
Aedes albopictus (dengue)	Oriental region; also in Celebes, Dutch East Indies, northern Australia (?), New Guinea (?), Hawaiian Islands (introduced)
Aedes scutellaris and closely related species (some or all may carry dengue)	Australasian region
A. hebrideus	Solomon Islands, New Guinea, Palau Islands, Dutch East Indies, Moluccas, Philippines, New Hebrides
A. pseudoscutellaris (bancroftian filariasis)	Samoa, Fiji, Ellice Islands, Marquesas Islands, Society Islands
A. tongae	Friendly Islands, Sikiana (Solomons)
A. andrewsi	Christmas Island
A. horrescens	Fiji
A. pernotatus	New Hebrides
A. guamensis	Marianas
A. hensilli	Carolines
A. alorensis	Lesser Sundas
A. paullusi	Sangir Island, Philippines
A. quasiscutellaris	Solomon Islands
A. marshallensis	Marshall Islands, Gilbert Islands
A. riversi	Okinawa, Ryukyu Islands

CONTROL. Control of these species is best accomplished by eliminating their breeding places. Since nearly all of the disease-bearing *Aedes* are domestic in their breeding habits, it is important to make weekly inspections of habitations and camps. Tin cans, bottles, jars, pails, old tires, sagging eave troughs, rain barrels, fire buckets, and cisterns are likely sites. *Aedes aegypti* is not commonly found in tree holes, differing from members of the *scutellaris* group and *A. albopictus* in this respect. When artificial containers cannot be eliminated, they should be screened or covered to exclude mosquitoes and inspected once each week to detect the presence of larvae. In open wells, water barrels, or cisterns, a light volatile oil, such as kerosene or nonleaded gasoline, will kill larvae quickly; these compounds readily evaporate, leaving the water suitable for drinking or washing purposes.



Anatomy of a culicine larva (*Culex quinquefasciatus* illustrated).

GENUS CULEX. *Culex quinquefasciatus* (syn. *C. fatigans*) is the common, brown, house mosquito and is an important pest in the warm temperate, tropic, and subtropic regions of the world. The adult is one of the important vectors of *Wuchereria bancrofti*. *Culex pipiens* is a vector of filariasis in Egypt, China, and Japan; it is a common house mosquito in the United States and has been shown to be a capable vector of *W. bancrofti* by Newton and Pratt (1946). *Culex tarsalis* has been found naturally infected with the western strain of equine encephalomyelitis and with human encephalitis in the United States. *Culex tritaeniorhynchus* is a known vector of Japanese B encephalitis.

There are about 300 species of mosquitoes in the genus *Culex*, and, like *Aedes*, they represent a very common group in most collections. The genus has been broken up into numerous subgenera, and frequently it is impossible to identify the females of the subgenera with any degree of certainty. Species of *Culex* are rather dull-colored, and the mesonotal ornamentation, as found in many of the

important *Aedes* (subgenus *Stegomyia*), is lacking. The larvae of *C. quinquefasciatus* breed by preference in artificial containers; those of *C. pipiens,* also a domesticated species, are found in rain barrels, cisterns, and polluted grassy pools and swamps.

CONTROL. Domestic species of *Culex* may be controlled by removing or overturning all water-holding containers; breeding in cisterns and wells can be reduced by screening, spraying with kerosene, or introducing surface-feeding fish.

GENUS ANOPHELES. Probably no other group of insects has received so much attention as the anophelines, for they alone are responsible for the transmission of malaria, the most important of all arthropod-borne human diseases. In some parts of the world, certain anophelines are also important vectors of filariasis (*Wuchereria bancrofti* and *W. malayi*). The recent work of Byrd and St. Amant (1945) indicates that *Anopheles farauti* is the important vector of *W. bancrofti* in the area of the New Hebrides and Solomon Islands in addition to being the primary malaria vector in that region.

Not all anophelines are natural vectors of malaria; Hackett and Russell (1938) state that of the 175 odd species which may be considered as potential vectors, the great majority are rendered harmless in nature because their biting habits do not bring them into frequent and, especially, repeated contact with man.

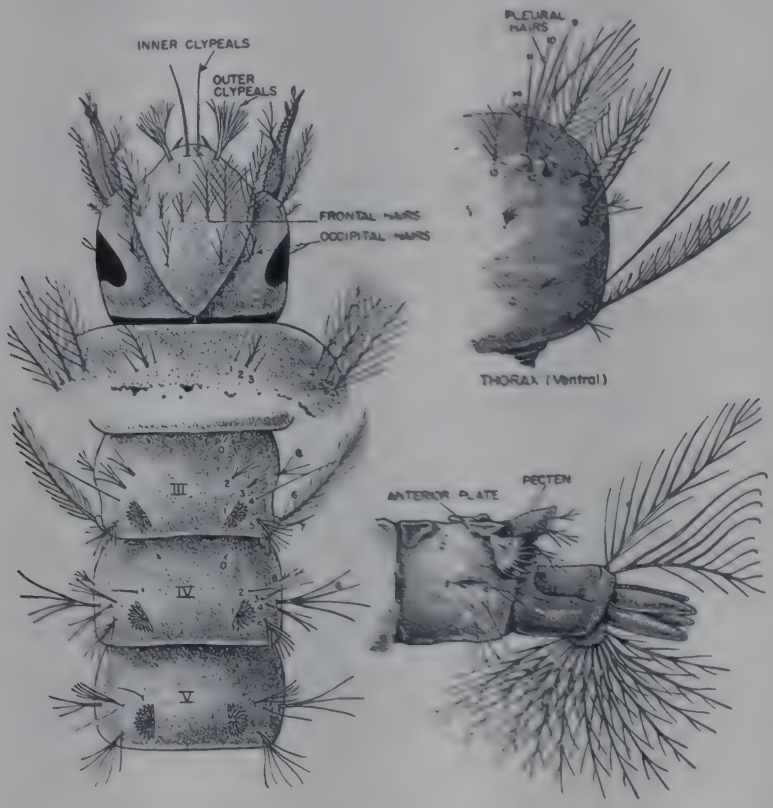
Among the factors that may be important in determining whether a given anopheline is an efficient vector of malaria or filariasis are: the type of breeding place or places and their distance from human habitations; the usual length of flight and the tendency of the adult mosquitoes to enter houses; the occurrence, abundance, and seasonal prevalence of the anophelines; their inclination to bite and their time of feeding; the susceptibility of the mosquito to infection and the choice of hosts (man or animals) as determined by precipitin tests; and the sporozoite or filarial index.

The great majority of the species conform to the commonly recognized pattern of "spotted-wing mosquitoes." Unlike the culicines, the females have maxillary palpi about as long as the proboscis, an important criterion in separating the two tribes. The scutellum is evenly rounded or crescent-shaped; the abdomen does not have scales on the first abdominal tergite (or at least the sternites are bare); and the legs are long and slender. The larvae have rudimentary breathing tubes and lie parallel to the under surface of the water. Palmate hairs are usually present, at least on the abdomen.

The life histories show much variation within the genus; it is generally felt that the time required for development is longer than that for most other genera. The life cycle of the anopheline mosquitoes under favorable conditions usually varies from 14 to 21 days, although some species may hibernate in the egg, larval, or adult stage. In the Tropics where breeding is relatively continuous throughout the year, the number of days required for completion of the aquatic stages may be materially reduced so that the life cycle may not take over 10 days. Most of the species probably prefer the comparatively pure, quiet, fresh water of pools, ponds, marshes, streams, and swamps; some prefer polluted water;

others may breed in the axils of water-holding plants; still others select swiftly flowing streams with or without marginal vegetation; a few are able to breed in brackish water.

The classification of mosquitoes is steadily undergoing changes which follow the progress of the science of medical entomology. The scope of the present discussion does not permit the inclusion of large keys necessary to identify the anopheline mosquitoes of the world. The following pictorial keys to the larval and adult anophelines will separate the several species occurring in the United States (see pp. 696, 697).



Anatomy of an anopheline larva (*Anopheles quadrimaculatus* illustrated). (Redrawn after Ross and Roberts.)

CONTROL. In planning mosquito control as an antimalarial measure, it is important to recognize the different species of *Anopheles* that are efficient vectors in the area concerned. As already stated, only a few species are active vectors of the disease; in the United States and Europe only two or three species are of primary importance. Different races of *A. maculipennis* are the most important in Europe as are *A. quadrimaculatus* and a variety of *maculipennis* in the United States. In India, Russell (1939) reports that about 13 species are still under suspicion.

There are three important methods in the prevention of malaria, all of which were combined in the earlier campaigns against this disease in the Canal Zone in Panama and more recently in the extensive control throughout the Pacific Islands: (1) Protection of the individual from bites of mosquitoes. (2) Destruction of adult mosquitoes and their breeding areas. (3) Chemical prophylaxis (Harper, Lisansky, Sasse, 1947). Of these three, only (1) and (2) apply to the mosquito vector and will be briefly summarized.

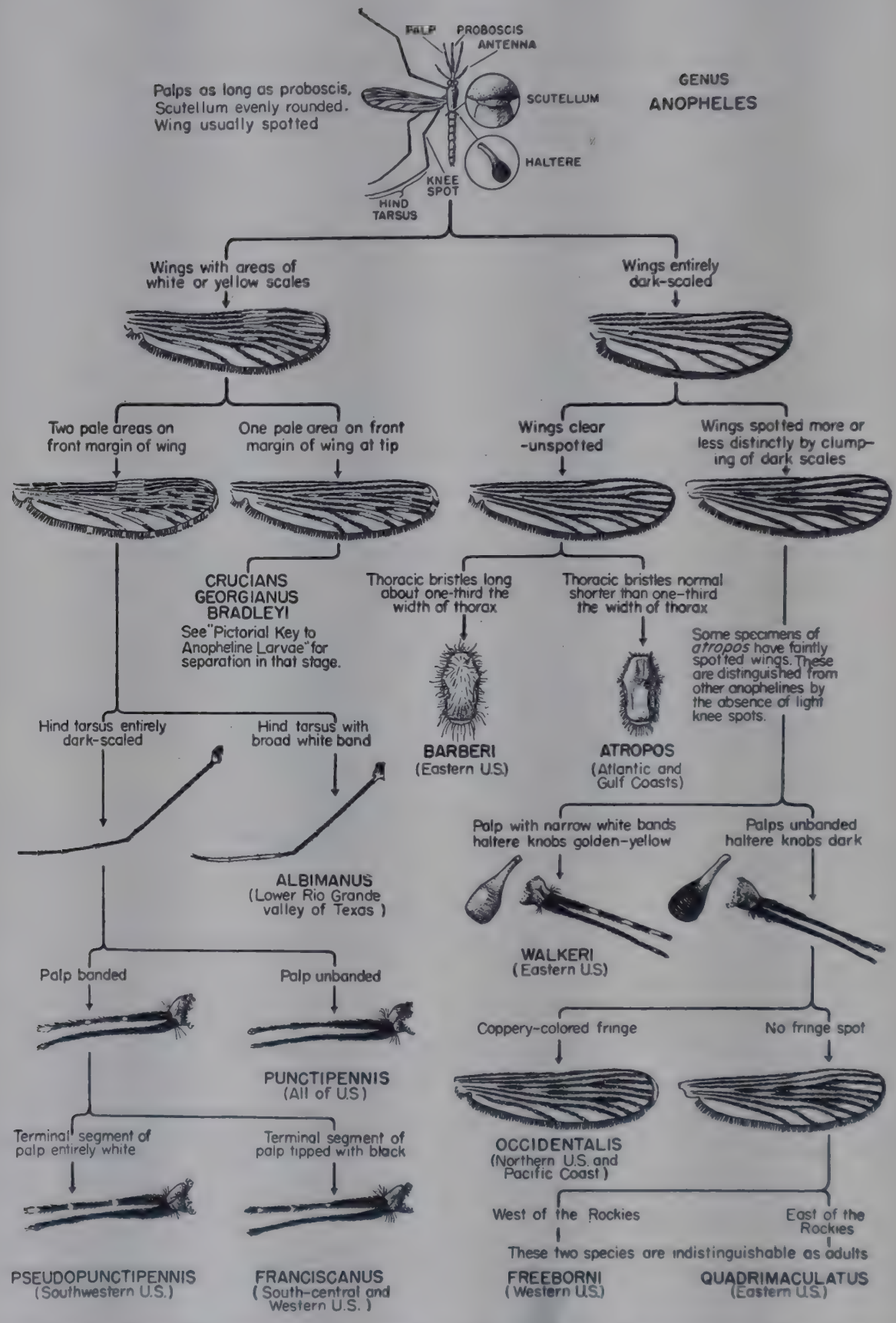
Protection of the Individual from Bites of Mosquitoes. These measures primarily include using bed nets, insect repellents, and proper screening (18-mesh); removing adult resting places; wearing proper clothing; avoiding malarious areas; and following other personal protective measures to preclude the passage of the malaria parasite from man to mosquito and from mosquito to man, thus interrupting the transmission cycle. Education regarding the dangers of malaria and the method of infection also has been of considerable importance in this respect; Russell (1941) has discussed some of the social obstacles to malaria control.

Destruction of Adult Mosquitoes and Their Breeding Areas. The basis of permanent prophylaxis against malaria in a given region must depend upon the destruction of the particular species of *Anopheles* which is involved in the spread of the disease in the region concerned. Control measures should be directed toward the larvae, pupae, and the fully developed insects. Against the adults, spray-killing with the various available insecticides will result in an appreciable reduction in transmission of the disease. The possibility of airplanes and ships introducing malaria into a country, by carrying infected anophelines from an endemic center, should not be overlooked. In all probability, *A. gambiae* crossed the ocean from Dakar, either by aircraft or fast destroyer, and was introduced into South America. This efficient vector resulted in transmission of malaria to over 90 per cent of the population in Ceara and Rio Grande Do Norte. According to the Rockefeller Foundation, mortality in certain districts amounted to 10 per cent; a subsequent report states that there were more than 5000 fatal cases. Such accidental introductions can be avoided if all vehicles from endemic centers are treated with pyrethrum sprays, both on arrival and on departure.

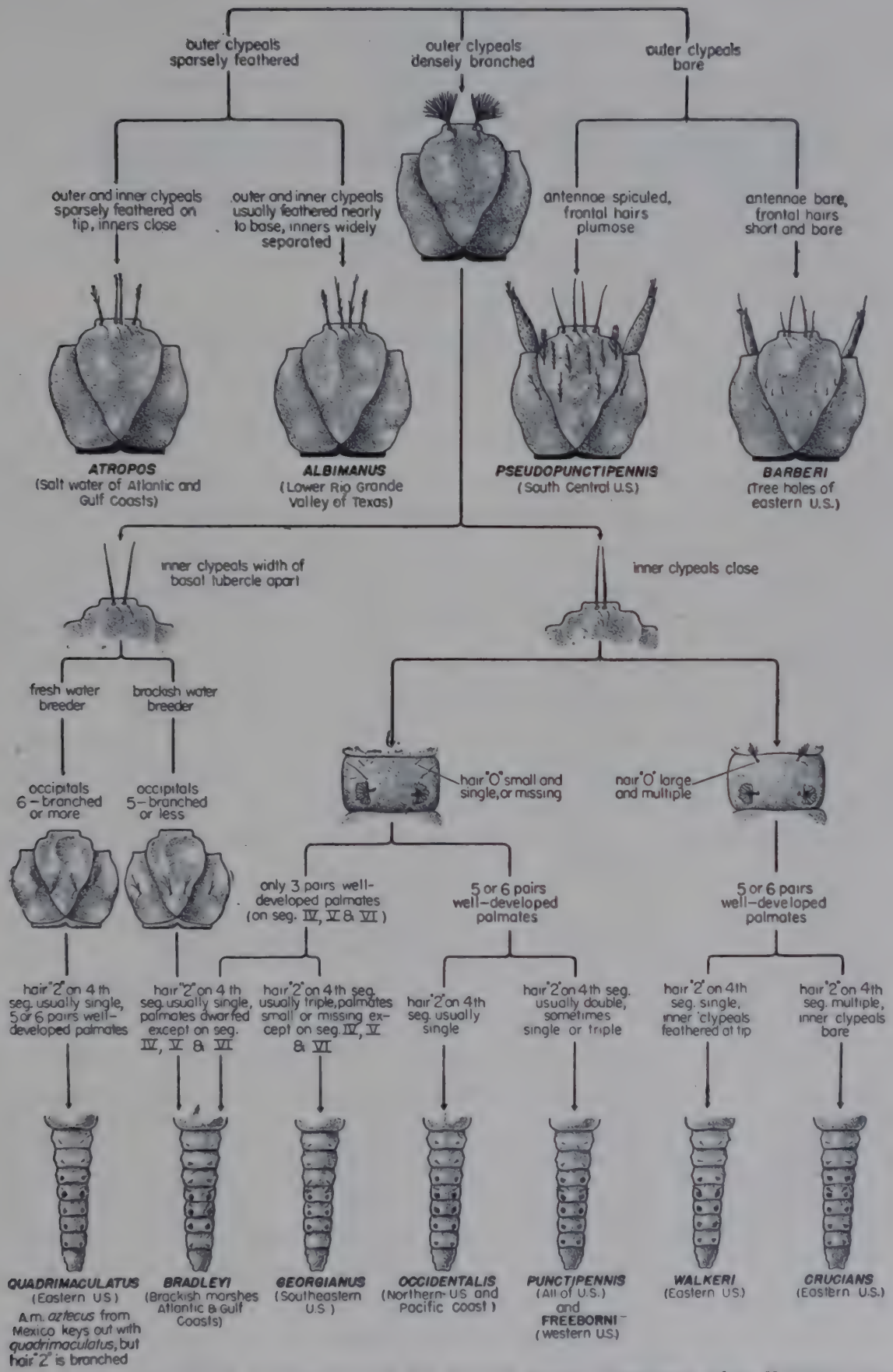
Against the larvae, permanent control measures are preferred to temporary ones. The elimination of mosquito-breeding areas may be accomplished by filling, draining, or stream-clearing. In different parts of the world, extensive sanitary engineering projects for permanent drainage have been carried out with considerable success, but such procedures are not feasible in many localities and little success has been obtained in others.

According to Watson (1940), on many estates in India much malaria is entirely attributable to man's interfering with nature by clearing away jungles and streams and exposing them to sunlight. By so doing, the harmless species have been driven out and the breeding of dangerous ones permitted, as *Anopheles minimus* in the north, *A. fluviatilis* or *A. culicifacies* at different elevations in the south. The elimination of "man-made" breeding areas of *A. farauti* (syn. *moluccensis*) in the New Hebrides-Solomon Islands region was one of the major problems in the control of this vector of malaria among military forces during World War II (Perry, 1946). Nearly 75 per cent of the breeding of this species was restricted to such artificial catchments as road ruts, improperly constructed roadside ditches, and similar areas.

Drainage should never be attempted without consultation of capable medical entomologists, field malariologists, and sanitary engineers. Faust (1937) points out that within recent years relief drainage, carried on without the supervision of



Pictorial key to the adult female anophelines of the United States. (Redrawn after Daggy, courtesy. U. S. Public Health Service.)



Pictorial key to the larval anophelines of the United States. (Redrawn after Knutson, courtesy, U. S. Public Health Service.)

sanitary engineers or entomologists, has increased the breeding places of *Anopheles quadrimaculatus* in the southern United States, with consequent outbreaks of the disease in previously nonmalarious areas. Especial attention has been devoted by the Division of Malaria Control of the Tennessee Valley Authority to the preparation of reservoirs before the impoundage, effort being made to present a clean water surface after filling. With the successful employment of fluctuation of water level as an antilarval measure, various accessory control procedures have been utilized more extensively. It has been shown that marginal drainage, shoreline improvement (drift removal), and herbicide work are essential to secure the maximum results from variation of water fluctuations.

When filling, drainage, or clearing is not practicable, the spraying of water surfaces with chemical larvicides is generally practiced. Paris green, diesel oil ⁂2, kerosene, or DDT are useful in controlling anopheline larvae. The methods of application and amounts to be used vary under different conditions; in most instances, directions for use are supplied with the organic larvicides. In airplane distribution of dusts or sprays, many problems are encountered.

More details in regard to the description of species of anophelines and their control as vectors of malaria are beyond the scope of this text. Information compiled from antimalarial campaigns, particularly on the experience gained by malaria control and survey organizations of the Armed Forces, is well recorded by Russell, West, and Manwell (1946). The important features of practical malariology, integrating the newer developments with older information, are therein presented, and an excellent account of the basic elements of each phase of the subject is clearly defined. Publications dealing with the use of DDT as an insecticide are available through the Bureau of Entomology and Plant Quarantine, U. S. Department of Agriculture, Washington, D. C.

Horse Flies, Deer Flies (Suborder Brachycera)

Of the recognized 17 families in this suborder, only one, the *Tabanidae,* is of medical importance. There have been occasional reports of some species in the family Rhagionidae biting man, but the habit is an exception rather than the rule. Many species of tabanids may act as mechanical vectors of disease, the organisms not developing or multiplying within the body of the flies. *Chrysops discalis,* the western deer fly, was shown by Francis and Mayne (1921) to convey *Pasteurella tularensis* in this way to man and animals. Tabanids have been suspected as vectors of espundia (*Leishmania brasilensis*) in the forested regions of Brazil and Paraguay. *Chrysops dimidiata* and *C. silacea* serve as the intermediate hosts of *Loa loa* in tropic Africa.

Biology and Morphology. Members of the family Tabanidae are usually large or medium-sized flies with robust bodies and large eyes. The antennae are porrect and variable in structure, always three-segmented; the third joint is often annulated. In most of the genera the antennae are short, although in members of the genus *Chrysops* they are longer than the head.

The eggs are deposited in masses on water plants and grasses overhanging



Chrysops discalis, the deer fly or green-head.

marshy areas. The carnivorous larvae are usually aquatic and are to be found in the muddy bottoms of swamps, ponds, and streams. Adult males live upon plant juices. In some of the genera—*Tabanus, Haematopota, Pangonia,* and *Chrysops*—the females are well-known for their blood-sucking habits.

Tabanus. There are over a thousand species in this genus. The three-segmented antennae are shorter than the head; ocelli are absent; the wings are usually clear, but spots and bands may be present; spurs are absent at the ends of the hind tibiae.

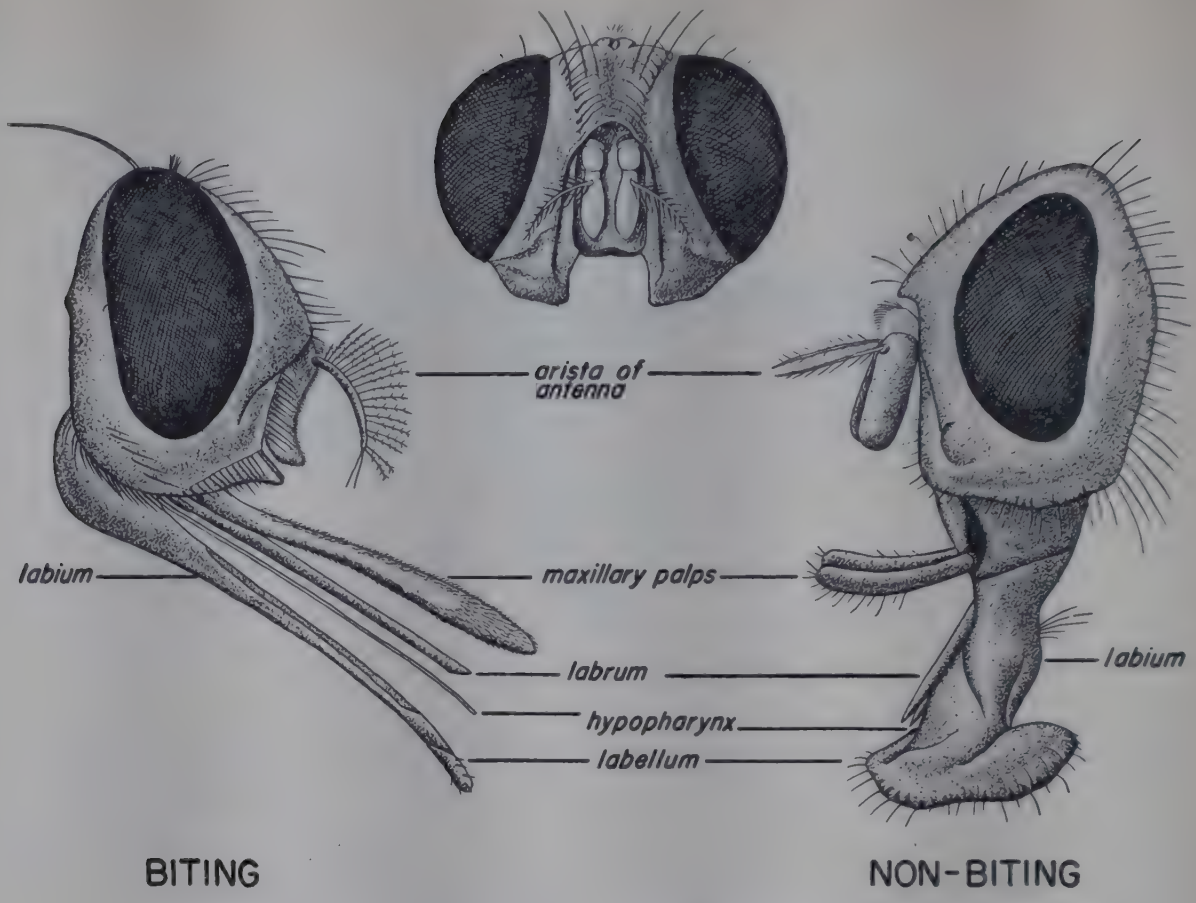
Chrysops. The conspicuous markings on the wings, the long antennae, and marked ocelli (three in number) are characteristic of this genus. The second segment of the antenna is as long as the first; the third segment has four annulations. The hind tibia has small spines at the tip.

Control. Effective control is difficult; drainage of swamps where tabanids are breeding has limited value. Repellents are not too effective. A hymenopterous insect which attacks the egg masses has been introduced into some sections of the United States and is giving promising results.

Tsetse Fly, House Fly, Green Bottle Fly (Suborder Athericera)

The members of this suborder comprise a large number of medium to small flies. They may be separated from members of the previous two suborders by their antennae which are rather short and never have more than three segments. The distal one bears an *arista* on its base. Dipterologists have broken this suborder into several families; for practicality and convenience the suborder is here divided into two categories according to mouth-part structure, and the terms *biting* and *nonbiting* flies are used to separate the medically important groups.

The *biting* forms have a rigid chitinized structure, the *labium,* which forms the piercing organ. This beaklike proboscis normally projects forward when not in use. The labium of the *nonbiting* forms consists of a rather flabby mem-



Mouthpart structure of biting and non-biting flies (suborder Athericera).

branous structure suspended from the lower part of the head which may be pulled up or extended at will. The broad, flattened, pincushion-like *labella* at the tip of the proboscis are not capable of piercing the skin. Spines and teeth on the labellar lobes enable a few species to scrape or even puncture the skin of man or animals.

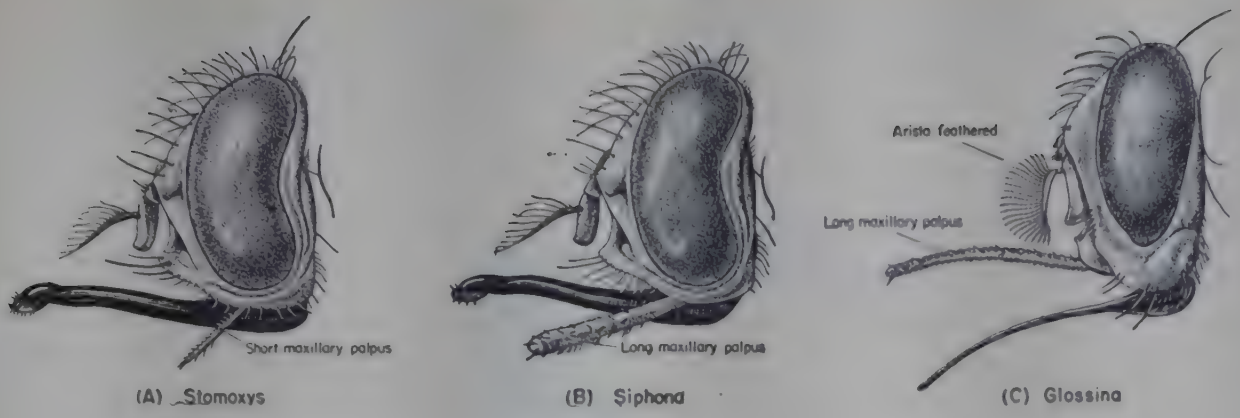
BITING FLIES

Stable Fly, Dog Fly. Members of the genus *Stomoxys* closely resemble the house fly and are erroneously referred to as "biting house flies." *Stomoxys* has frequently been used in experimental transmission of disease, but there is no conclusive evidence that it is a vector in nature. Larvae are found breeding in decaying piles of hay, straw, lawn clippings, and in cast-up seaweed along beaches. They are known to breed in animal wastes but never in human excrement. Macgregor (1945) has reported a case of accidental intestinal myiasis caused by ingesting food contaminated with eggs of this species.

Horn Fly (Genus *Siphona*). This is a serious pest of cattle and occasionally attacks man. It closely resembles the house fly, but is only about half as large.

Tsetse Fly (Genus *Glossina*). This genus is limited to tropic Africa and includes about 25 species. These flies are peculiar in that the female is viviparous, producing a single, very large, fully developed, yellowish-brown, motile larva. The larvae are deposited in shady, sandy soil. Pupation takes place almost immediately.

Glossina palpalis is the principal vector of West African sleeping sickness (due



Heads of three important biting flies (suborder Athericera).

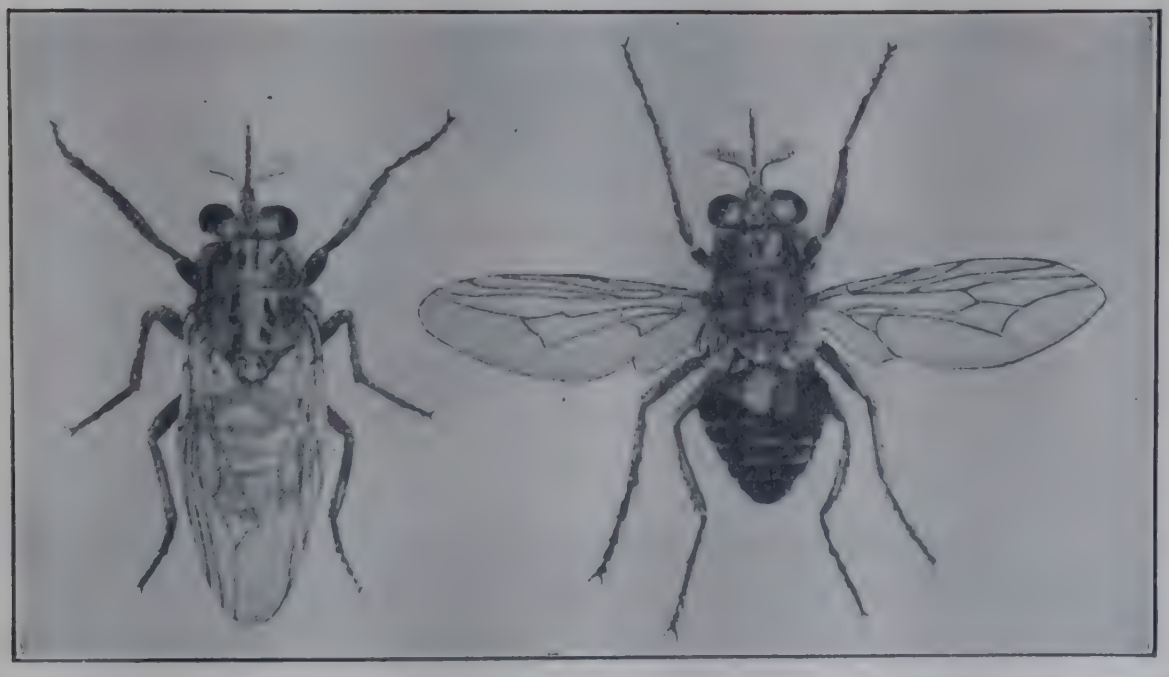
to the parasite *Trypanosoma gambiense*); *G. morsitans* is the transmitter of the East African form of the disease (due to *T. rhodesiense*) in Rhodesia, the Sudan, and the Belgian Congo. *Glossina tachinoides,* which Lester (1936) reported to be more frequently infected with *T. gambiense* in nature than *G. palpalis,* is found in a belt along the southern border of the Sahara from the Atlantic to Arabia.

Control. The control of tsetse flies is difficult considering the long flight range of adults and the habits of the female in depositing larvae. Known fly belts should be avoided; screened quarters and the wearing of white clothing are the best prophylactic measures.

NONBITING FLIES

The dividing line between families of this group is not always clearly defined; some genera have been shifted from family to family, while others are still awaiting permanent assignment to their correct group. It is impossible to discuss the species in detail; only the more common and important genera will be considered.

The house fly and many of its relatives are common agents in the mechanical transmission of certain infections which are often grouped under the term "fly-



Glossina palpalis in natural resting position and with wings outstretched. (MacNeal, after Doflein.)

borne diseases." The spongelike mouth parts, the numerous body spines, and the sticky pads on the feet of these flies have been found to carry a large number of different pathogens. Some of these disease organisms may pass unaltered through the digestive tract and remain viable in the feces or "fly specks." The method of feeding has an important bearing on the house fly's ability to transmit disease organisms. The secretions regurgitated during this act may harbor typhoid and cholera bacilli, or contain the pathogens causing amebic or bacillary dysentery which are thus transferred to food and milk. In the same manner, the spirochetes of yaws may be transferred by flies feeding on the ulcer and then depositing the organisms on scratches or skin abrasions of healthy individuals; likewise, the causative agent of trachoma may be spread by flies feeding on soiled bandages or on infective matter in the eyes of patients. Members of the genus *Hippelates* (the eye gnat) are attracted to man to feed on lacrimal secretions, open sores, cuts, and ulcers. Herms (1926) reported them as being very common in the Coachella Valley in California where they were important transmitters of organisms causing various types of conjunctivitis.

It should be emphasized that these filth flies may easily carry the pathogens found in excrement, sputum, open sores, or putrefying matter to food, milk, healthy mucous membranes, or to uncontaminated wounds.

The larvae of many of the genera may accidentally become internal parasites of man, producing a condition called *myiasis*. Clinically, the types of myiasis may be classified according to the part of the body invaded. Thus, when the invasion involves the intestinal tract, it is referred to as "intestinal myiasis"; when it involves the skin, "cutaneous myiasis." Other types are urinary, ophthalmic, auricular, and nasal myiases.

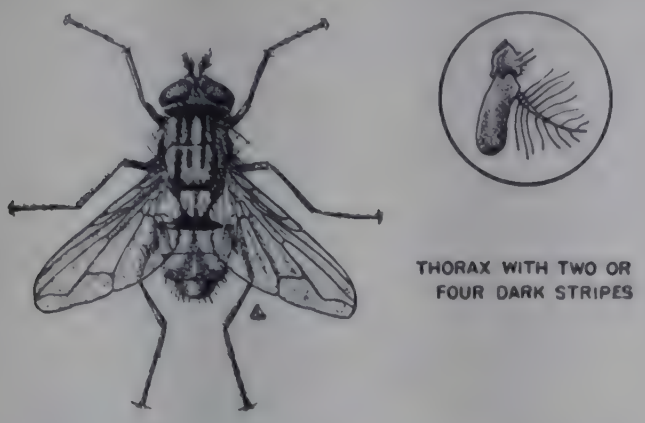
Musca Domestica. The common house fly is the most familiar representative of the order Diptera. The eggs are laid in masses of 75 to 150. A single female is able to lay as many as 21 batches in a single month after emergence.

Control. House-fly control is mainly directed toward the suppression of fly breeding, but also includes measures against adults, as well as protective devices designed to exclude flies and prevent them from contaminating food.

Manure, particularly that of horses and hogs, constitutes one of the principal breeding places, and its proper disposal is of great importance. It can be sprayed with a DDT-water emulsion in concentrations as low as 0.1 per cent to kill any fly larvae which may develop. Fly breeding in human excreta is particularly dangerous; wherever possible, toilets should be carefully fly-proofed. In temporary camps the use of trench latrines and prompt covering of feces are extremely important in reducing breeding. Applications of DDT to the feces for larval control is neither economical nor effective. Sodium arsenite is a powerful and cheap poison and 1 gallon of the concentrate, mixed with 40 to 80 gallons of water, will give excellent control for flies and fly maggots. Paradichlorobenzene, if available, is effective only if used in deep pits which are well sealed.

Neglected garbage furnishes excellent breeding material. Refuse cans should be available, kept tightly covered, and thoroughly cleaned when empty. Garbage can

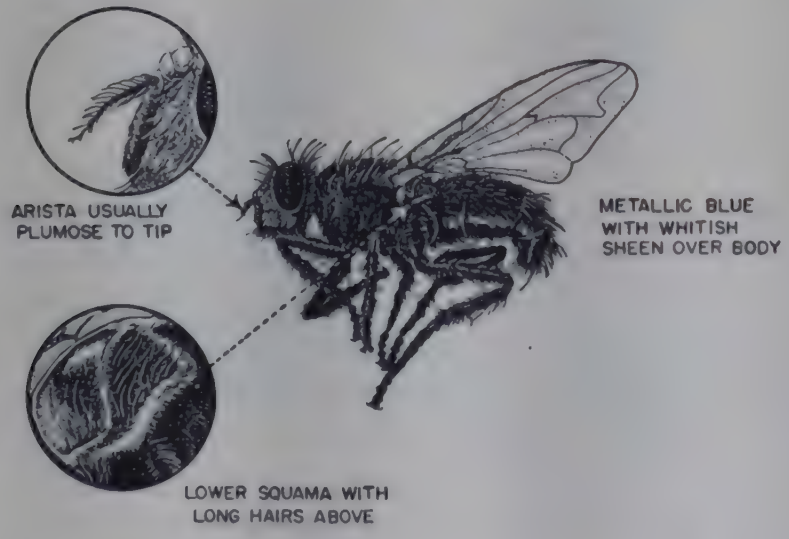
be burned, buried, or disposed of at sea some distance off shore. Screens, flytraps, sprays, poisons, flypapers, and flyswatters are all important measures against adults. Baited traps should be placed near galleys, mess halls, garbage racks, and



Musca domestica, the common house fly. (Courtesy, Dr. S. P. James, U. S. National Museum.)

latrines. The use of DDT as a residual spray is probably more effective against adult flies than against any other group of insects. A 5 per cent solution in kerosene or water emulsion should be applied just short of dripping to the surface where flies tend to concentrate. Screens should be hand painted with this solution.

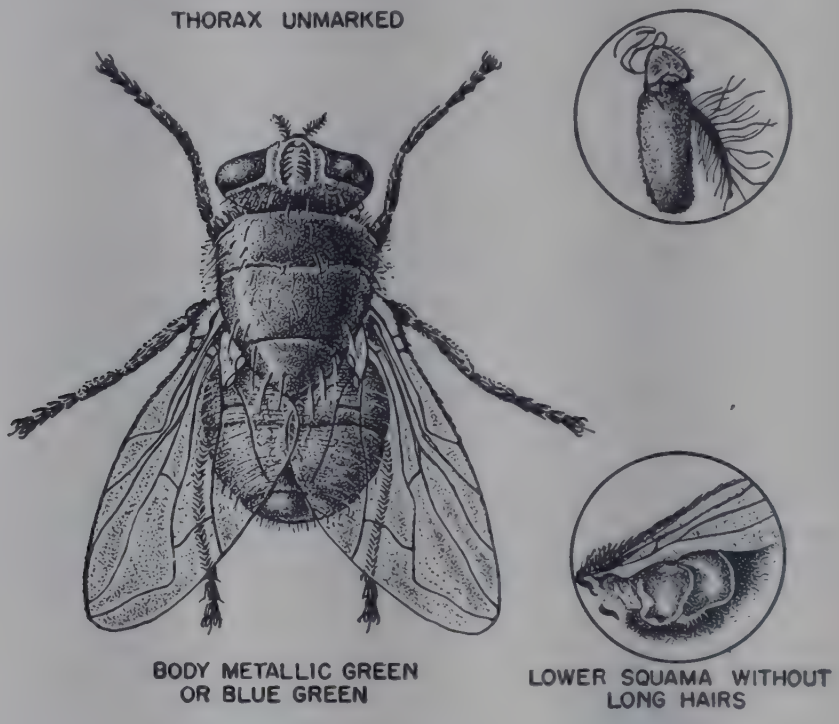
Calliphora. Members of this genus are rather large, about 10 to 15 mm. long, and are frequently called "blow flies" or "blue bottle flies"; they have red eyes; the body is blue in color and exhibits a slight metallic luster. *Calliphora erythro-*



Calliphora erythrocephala, the blue bottle fly.

cephala (red cheeks with black hairs) and *C. vomitoria* (black cheeks with red hairs) are two widely distributed species which are common in most parts of the world. These forms normally deposit ova on exposed food or decaying animal or vegetable matter of any kind, as well as in open wounds or ulcers of animals and, occasionally, of man.

Lucilia. Members of this genus are small, about the size of the house fly. They are world-wide in distribution with habits somewhat like *Calliphora.* The adults are usually metallic green in color, often being called "green bottle" flies. *Lucilia sericata,* distinguished by its yellow palpi, is a frequent cause of intestinal myiasis; *L. caesar* is less actively parasitic. The larvae of both species (and also of *Phormia regina,* the wool maggot or black blow fly) have been used extensively in the treatment of osteomyelitis, although all are capable of injuring healthy tissue. Stewart (1934) has particularly shown that *L. sericata,* which has been widely used as a surgical maggot because it appeared to be exclusively saprophagous, will attack and invade healthy living tissue.

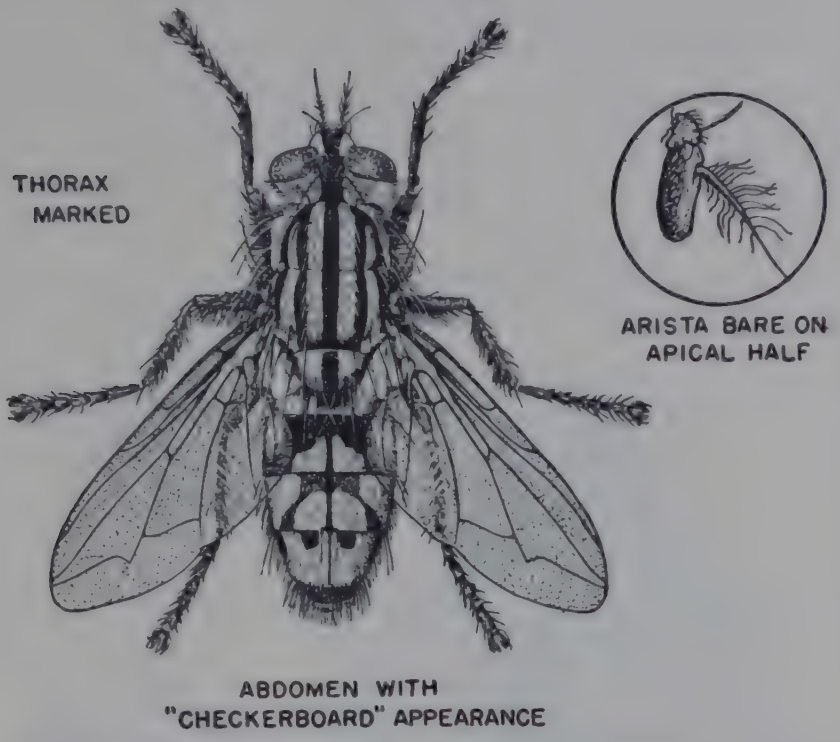


Lucilia sericata, the green bottle fly.

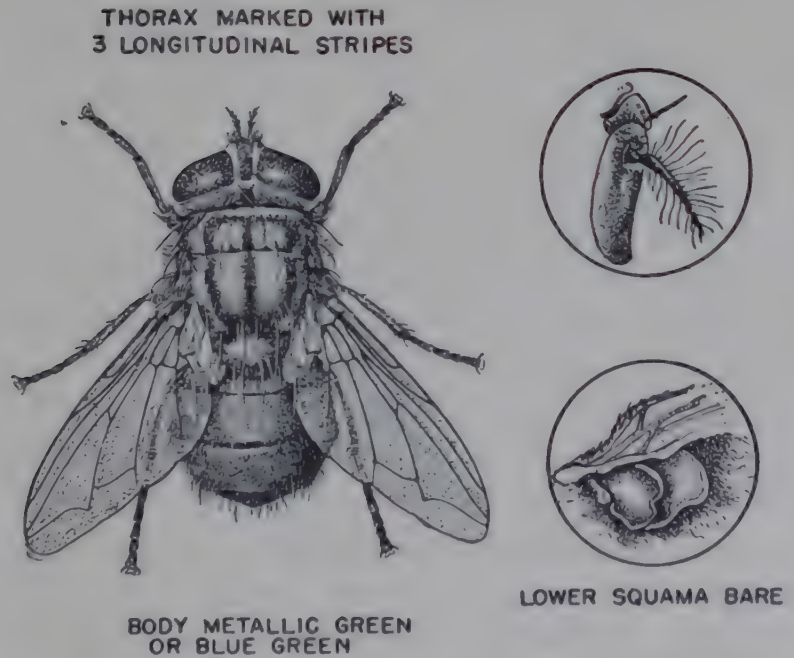
Sarcophaga. Species belonging to this genus are usually distinguished by their rather large size. The thorax is striped and the abdomen commonly marked with dark areas in a "checker-board" fashion. The adults, commonly called "flesh flies," are frequently observed about garbage and decaying animal matter. The larvae have been recorded living as parasites in sores and in the nasal cavities and intestinal tracts of man and animals. The genus is peculiar in that some species are viviparous. The larvae have powerful, curved mouth hooks; the stigmal plates are set in a deep cavity at the posterior end of the body.

Cochliomyia. The adults of this genus superficially resemble the green bottle flies (*Lucilia*) but may be recognized by the three longitudinal dark stripes on the thorax. Two species, *C. americana* and *C. macellaria,* are of particular interest, for the larvae are known to be important producers of several types of myiases. *Cochliomyia americana* is found in the southwest United States and throughout the American tropics. *Cochliomyia macellaria* is frequently found in association with *C. americana,* but it appears to be a secondary invader. Eggs of *C. macellaria*

are often deposited in carcasses of animals; the larvae normally confine their activities to necrotic tissues.



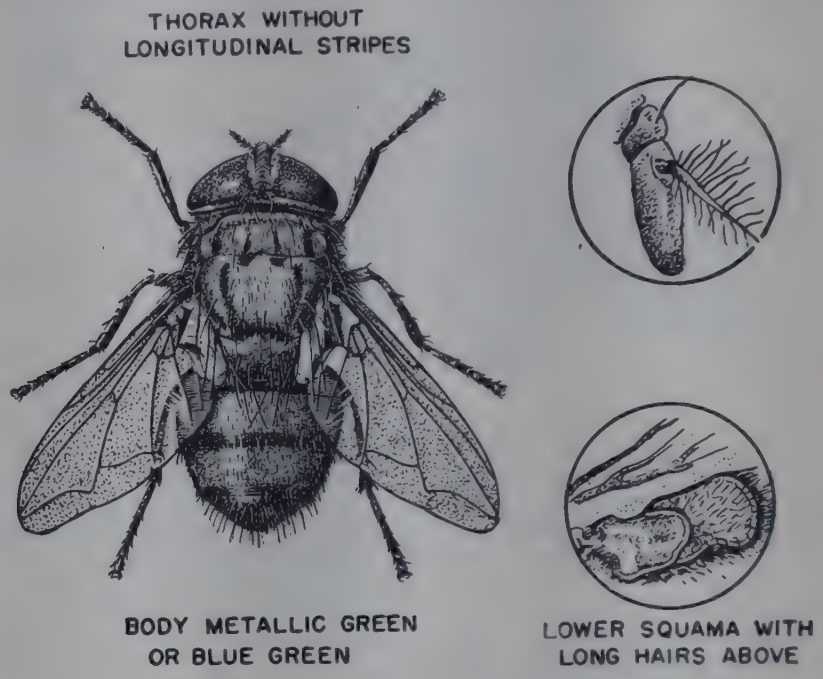
Sarcophaga haemorrhoidalis, the flesh fly. (Courtesy, Dr. S. P. James, U. S. National Museum.)



Cochliomyia americana, the primary screw-worm fly. (Courtesy, Dr. S. P. James, U. S. National Museum.)

Musca Sorbens. Throughout the Central and South Pacific Islands this fly has become the most objectionable pest in and about camp areas. It breeds primarily in cow dung and horse manure and occasionally in human excrement. It is readily attracted to open sores and wounds and in Mesopotamia it is known to be an important transmitter of various types of conjunctivitis.

Chrysomyia. These flies resemble *Cochliomyia* but may be generally separated by their different geographic distribution. The genus is confined to Africa, islands of the Pacific (including the Philippines), Australia, and certain sections of Asia. They are medium-sized flies of a light metallic green or blue-green color. The larvae are frequently reported as parasites of sheep and are known to cause a disfiguring myiasis of man. The adults are readily attracted to bleeding surfaces and will oviposit in the ears and nostrils of persons having offensive discharges.



Chrysomyia bezziana, the Old-World screw-worm fly. (Courtesy, Dr. S. P. James, U. S. National Museum.)

Wohlfahrtia Magnifica. The larva of this species is a common cause of cutaneous myiasis in the Old World. Its habits are like those of *Cochliomyia americana.* The larvae are deposited on the skin or about the eyes and, at least in young children, they may penetrate the unbroken surface. Several cases of myiasis due to *W. vigil* have been reported in North America.

Dermatobia Hominis. This is a large, thick-set fly about 15 mm. long, with prominent head and eyes, small antennae, and a marked narrowing at the junction of the grayish thorax and metallic-blue abdomen. The species is widespread in tropic America, living in damp forested regions. When ready to oviposit, the flies capture *Psorophora* mosquitoes as they emerge from the pupa (and occasionally other biting or nonbiting Diptera or even ticks) and deposit 15 to 25 eggs on the ventral side of the abdomen, gluing them to it in such a position that the point of emergence of the larva is directed away from the mosquito. When the mosquito contacts either man or other warm-blooded animals in the process of biting, the larva is stimulated to emerge; it then burrows through the puncture wound into the subcutaneous tissue by means of its powerful mouth hooks. Here it develops into a club- or flask-shaped structure (ver macaque), later becoming more cylindrical (called "torcel" in Venezuela, and "berne" in Brazil). It is girdled by several rows of prominent spines. As the larva grows, a tumor-like

swelling develops with a central orifice toward which the posterior (pointed) extremity of the larva projects and through which it takes air into its spiracles. After a period of 7 to 15 weeks, it leaves the tissues and burrows into moist soil to pupate. Harrell and Moesley (1942) point out that the larvae may at times burrow deeply into vital tissues.

Auchmeromyia Luteola. The larva of this species is the blood-sucking "Congo floor maggot." It is about 16 mm. long and has a dirty-white, thick, leathery, wrinkled skin; otherwise, it resembles the larva of *Musca domestica.* The adult fly deposits her eggs by preference in the dry dust of cracks on the floor of native huts. The larvae hatch in a few days and seek blood within a few hours, crawling out at night to feed on sleeping natives. They are said to survive for a month without food. This is the only known instance of a blood-sucking larva which attacks man. (Other species of blood-sucking larvae infest the nests of birds and the burrows of certain mammals). Attacks of these maggots may be avoided at night by sleeping on a cot a few inches above the floor.

Cordylobia Anthropophaga. The larva is known as the "African skin maggot," or "ver du Cayor." The ova are deposited in dry sand, occasionally on clothing, but not directly on the skin. After three or four days the larva emerges and by means of its mouth hooks attaches itself to the skin of the first animal with which it comes in contact, most often a rat or a dog, but quite frequently a child. The larva bores its way painlessly into the skin and produces a lesion like a boil which has a central opening through which the larva breathes. It resembles the ver macaque, is somewhat barrel-shaped, and beset with small spines. Twelve to 14 days after penetration it leaves the body of the host and pupates in the soil.

Gastrophilus Intestinalis. This species is commonly known as the "horse bot." A number of cases of human infection have been reported; however, man appears to be an unsuitable host. When the larvae reach the skin they tend to migrate aimlessly and give rise to a "creeping eruption" similar to that caused by the larvae of the dog hookworm. Like *Dermatobia,* mouth parts are vestigial in the adult stage.

Eristalis Tenax. The larva of this species has been reported to cause intestinal myiasis. It is popularly known as the "rat-tailed" larva because of its long breathing tube. Schwartzwelder (1942) reports 22 cases of human infection; all but one involved the gastrointestinal tract. Immature forms are found in polluted waters.

Piophilia Casei. The small black, rather antlike fly, commonly known as the "cheese skipper," has been reported by Simmons to be a common cause of intestinal myiasis. Cheese, bacon, ham, smoked meats, and fish are especially favored by the larvae. Man becomes accidentally infected upon eating these foods.

Determination of Dipterous Larvae

Certain points in the anatomy of dipterous larvae must be considered in determining genus, family, or even species. Maggots, the larvae of flies, are footless, wormlike, and more or less cylindrical. The broad extremity is usually the posterior end and the tapered one the anterior or head end. The terminal seg-



Body structure and stigmal plates of some myiasis-producing fly larvae.

ment bears two chitinized plates; these are the posterior stigmal plates; their shape, sculpturing, and position are characters used in specific determination. Detailed preparation is not necessary for an examination of the plates and spiracles. It is sufficient merely to remove a thin slice at the posterior end and to examine the exterior surface under the low-power objective of the microscope.

Collection and Preservation of Arthropods

Specific identification of medically important arthropods is often very helpful or even essential in determining which measures should be used in their control. Methods of collecting and preparing such specimens for identification are described in the pages immediately following.

Live specimens should not be sent through the mails. In shipping specimens mounted on slides or contained in vials of alcohol, special care must be taken in packing to prevent breakage. In the Tropics, particular care should be taken to store insects in dry containers. Mold and insect pests will soon destroy a collection if proper care is not taken. Salve tins or pill boxes, packed with adults, as indicated below, should be enclosed in mailing tubes or other sturdy containers for shipping. If mounted adults are sent, the pins must be forced firmly into place and the mounting box must be enclosed in excelsior within another sturdy shipping box. All shipped material should be labeled "Fragile" and accompanied by complete data as to locality, date, elevation, collector's name, and other pertinent information as to habits, habitat, abundance, and distribution. As far as possible, reared specimens should be accompanied by associated larval and pupal skins.

Mosquitoes. Adult mosquitoes are usually collected at catching stations, in bait or light traps, or from various daytime resting places. A chloroform tube is commonly used in their capture. Such a tube can be easily prepared by placing a half inch of cut rubber bands or other rubber scraps in the bottom of a large shell vial or test tube, saturating the rubber with chloroform, covering with a plug of crumpled paper or cotton, and topping with a circle of stiff paper. Various types of suction apparatus are used for taking specimens alive or in large numbers.

Minuten nadeln may be used to pin freshly killed adults. The adult mosquitoes may also be glued to paper points, using Duco Household Cement, orange shellac, or some other adhesive. Dried specimens should be relaxed before being mounted; care should be taken not to rub the specimens or break off the more fragile body parts.

Unmounted adults can be placed between layers of glazed cotton, cellucotton, or cleansing tissue in pill boxes or salve tins of appropriate size. Plain cotton is objectionable because the specimens become entangled in the fibers and breakage results when they are removed for mounting. The cellucotton expands and contracts, depending upon the humidity; therefore, care should be taken to cut the sections of this material large enough to fit snugly to allow for shrinkage when drying. If naphthalene or paradichlorobenzene is added to prevent the development of mold or the attack of insect pests, it should be used sparingly in fine crystals in the bottom of the pill box (preferably in the outside container) and should not be allowed to come in contact with specimens.

Terminalia. A study of male terminalia is often necessary for specific identification. The terminalia must be cleared and mounted on slides for microscopic examination. Many methods of preparation are known; a simple one is given below.

1. Clip the tip of the abdomen with fine scissors and wet in a dish of 70 to 95 per cent alcohol.

2. Transfer with a pipet or a bent needle to a dish of 10 to 20 per cent potassium hydroxide for 5 to 20 minutes—longer if the specimen is to be stained.

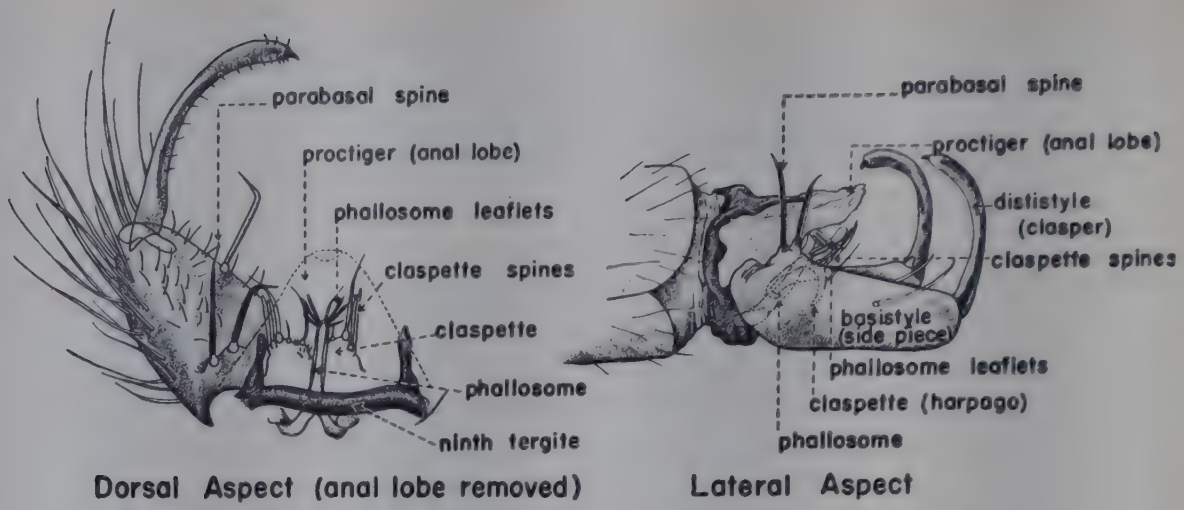
3. Transfer to a slide and remove the excess potassium hydroxide by blotting from the margin of the drop.

4. Add a small amount of glacial acetic acid to neutralize the potassium hydroxide and partly dehydrate; blot off the excess.

5. Add a drop of chloral-gum media, described under larval mounts; orient the specimen and place small bits of glass or paper around it to prevent crushing by the coverslip.

If balsam or clarite mounting media are desired, the specimens should be cleared by adding a drop of clove oil or xylol between steps 4 and 5, and the clarite or balsam substituted for chloral-gum in step 5.

In the genus *Anopheles* the proctiger tends to obscure the details of the phallosome and claspettes; it is desirable to dissect these from the proctiger and ninth segment. This should be done under a dissecting microscope just before adding the mounting medium. For a special technic on dissecting and staining *Anopheles* terminalia, see Komp, Public Health Reports, Vol. 57, no. 36, p. 1327.



Anopheles male terminalia. (Lateral aspect redrawn after Ross and Roberts.)

Eggs. Fertile anopheline eggs can often be obtained by confining females in a small cage over a dish of water, or in a small vial with a few milliliters of water in the bottom. The eggs can be easily recovered by filtering, and the sheets of moist filter paper with the eggs can be packed between layers of damp cotton in a container sealed with paraffin. Eggs packed in this way will remain viable for several days and can be transported to a central laboratory for rearing. A sample batch of eggs on a narrow strip of moist filter paper can be preserved in formaldehyde fumes in a tube tightly corked and sealed with paraffin. A cotton plug saturated with 10 per cent formalin should be placed in the bottom and another dry plug should be placed about 1 cm. above it, so that the eggs are not directly wetted with formalin. The novocain tube is very satisfactory for this method of egg preservation.

Larvae. Mosquito larvae are usually found floating at the surface of the water where they can be collected by skimming the water with a cup or dipper. The larvae are removed with a wide-mouthed dropper or spoon, placed in collecting jars, and returned to the laboratory for rearing, identification, or preservation. They can be reared on scrapings of dog biscuit, pablum, yeast, or crushed toast crumbs, care being taken to change the water frequently. For quick identification the examination of a freshly killed larva in a drop of water on a slide is often sufficient. Details are much more readily seen when the specimen is cleared and permanently mounted on a slide. For careful study all mosquito larvae should be prepared in this way.

Before mounting or preserving mosquito larvae in alcohol, the specimens should be killed in a manner that avoids shrinkage and distortion. Single larval specimens in a drop of water can be killed by dipping them in hot (not boiling) water for 15 to 20 seconds. For storing, specimens should be passed through 50 per cent, then 70 per cent alcohol, and placed in vials with cotton plugs to prevent movement and breakage. Very convenient containers can be made from empty novocain tubes discarded by dentists.

Mounting media. A number of different media and technics may be used for mounting mosquito larvae and other small arthropods for microscopic study.

Chloral-gum Media. The use of Berlese's formula, or one of its several modifications, is advantageous since no dehydration of the specimen in alcohol is necessary. This reduces handling of larvae to a minimum and lessens the likelihood of mechanical injury. Some difficulty, however, has been experienced with the permanency of these mounts. Small fragments of glass or narrow strips of paper, previously saturated in the medium, can be placed on either side of the specimen to prevent crushing by the coverslip. The last few segments of a culicine larva should be nicked with a needle, but not completely severed, to allow the breathing tube to appear in full lateral view. The slide should be kept in a horizontal position for several weeks until the medium hardens. The edges

of the coverslip should then be ringed with some sealing agent such as clarite, isobutyl methacrylate, cellulose cement, black asphaltum sealing material, or Duco Household Cement.

Gater's modification of the chloral-gum formula is commonly used. It can be prepared as follows:

Gum arabic (gum acacia)	8 Gm.
Distilled water	10 ml.
Chloral hydrate	75 Gm.
Glycerin	5 ml.
Glacial acetic acid	3 ml.

The gum arabic is dissolved in water, the action hastened by keeping the water warm, and the other ingredients are added in the order given. The thick solution can then be strained through several thicknesses of clean muslin, if necessary. Other modifications have been recommended, but the above formula seems the simplest to prepare and its ingredients are available in most laboratories.

Polyvinyl Alcohol-Phenol-Lactic Acid Medium. The following medium provides another rapid method for mounting mosquito larvae and other small arthropods without clearing and dehydration in alcohol:

Polyvinyl alcohol stock solution	53.5 ml.
Phenol	25.0 ml.
Lactic acid	21.5 ml.

A stock solution is prepared by dissolving the powdered alcohol (Dupont-Grade RH-349A) in water until the solution becomes as viscous as thick molasses. This stock solution becomes clear on standing, or clearing can be hastened by heating over a water bath. Specimens can be mounted in this medium as described for chloral-gum, and the coverslips ringed for more permanent slides.

Canada Balsam, Clarite, and Isobutyl Methacrylate. For mounting specimens in media which have xylol as a solvent it is necessary first to dehydrate in 50, 70, and 95 per cent alcohol. The specimens should then be cleared in clove oil, carbolxylol (three parts xylol and one part melted phenol crystals), or absolute alcohol followed by xylol. The specimens should remain in the various changes 5 to 20 minutes. After clearing, they may be mounted in balsam, clarite, or isobutyl methacrylate. With balsam and clarite, hardening of the media may take several days. Isobutyl methacrylate, on the other hand, dries very quickly and slides can be used in a few hours; ringing or sealing is unnecessary. All slides should be fully labeled with locality, date, and collector.

Other Nematocerous Diptera. Specimens of the smaller diptera of medical importance —Ceratopogonidae, Psychodidae, and Simuliidae—are best preserved in 70 per cent alcohol in the field. *Phlebotomus* adults can be mounted later on slides for careful study, but if *Culicoides* are mounted in this way it is often difficult to make out the characteristic color markings on the wings. Simuliid adults can also be preserved in alcohol and later dried for mounting. In all three of these groups, specimens may also be preserved dry, as described for mosquito adults, and later mounted in various ways for more detailed study.

Mites. Specimens may be preserved in alcohol and subsequently mounted on slides. For temporary mounts, they may be pipetted onto a glass slide and covered with a drop of glacial acetic acid and gently heated over an alcohol lamp. This results in perfect clearing and extension of the specimen so that the finest details of both dorsal and ventral surfaces can be seen.

For permanent mounts, specimens are transferred to a drop of glacial acetic acid, covered and heated very gently until bubbling begins. The coverglass is then lifted off,

the specimen transferred to another slide and mounted in chloral gum or other suitable media. To prevent crystalization the acetic acid in the chloral gum technic should be replaced by the less volatile and more viscous glacial lactic acid.

Ticks. These arachnids can be picked from their host animals or they can be collected by "flagging"—drawing a rough flannel cloth over the grass and shrubbery in an infested area, and then removing any ticks that attach to the "flag." They may be preserved in 70 per cent alcohol or cleared and mounted on slides. If slides are to be made the ticks can be fixed in an extended position by pressing them gently between two glass slides while they are killed by dipping them in hot water. They can be stored in 70 per cent alcohol, cleared in potassium hydroxide, dehydrated in alcohol, and mounted in balsam, clarite, or isobutyl methacrylate, as described for fleas.

Fleas. These insects may best be collected from small animals by etherizing the host in a bell-jar or other large container and picking up the stupefied fleas that attempt to escape. If the host is killed, it should be dropped immediately into a tight cloth bag to prevent the escape of the fleas that leave the animal as soon as the body temperature begins to drop. Dogs or cats may be dusted with ground pyrethrum or derris root, and the fleas picked up from papers spread on the floor under the animal. Specimens may be preserved in 70 per cent alcohol or mounted on slides for specific identification by the following procedure:

1. Drop living fleas or preserved specimens into 10 per cent potassium hydroxide and allow them to remain there for a day or two until cleared sufficiently.
2. Transfer the specimens to water in a watch glass containing a few drops of hydrochloric acid; allow them to remain one-half hour.
3. Dehydrate in 50 per cent alcohol.
4. Place in 95 per cent alcohol for one-half hour.
5. Clear in beechwood creosote for one hour, or run through several changes of absolute alcohol and clear in clove oil or xylol.
6. Mount on slides in balsam, isobutyl methacrylate, or clarite.
7. Label fully, including host animal, locality, date, and collector's name.

Miscellaneous Arthropods. Spiders, scorpions, centipedes, millipedes, lice, bedbugs, maggots, nymphs, and other soft-bodied insects may be preserved in 70 per cent alcohol; the corks of the vials should be paraffined to prevent loss through evaporation. If a small amount of glycerin is added, the specimens will not become dry and shrunken upon loss of alcohol. The vials should be kept upright so that the corks will remain dry and stay in good condition for a long period of time. The larger, hard-bodied adult insects may be pinned and labeled carefully and stored in Schmitt or cigar boxes. Care should be taken to keep specimens dry. A small amount of naphthalene flakes or paradichlorobenzene should be added to storage boxes to prevent mold and damage by insect pests.

Fixing Fluid. The following formula provides an excellent fixing fluid for the study of insect anatomy. It is a clearing and relaxing fluid and works equally well for cleaning slides, coverslips, and other small glassware. The insects to be studied can be placed in the material for 30 minutes or less, depending upon the specimen.

Alcohol	265 ml.
Ethyl acetate	95 ml.
Benzol	35 ml.
Water	245 ml.

The ingredients are mixed in the order named.

Technic for Determining Source of Mosquito Blood Meals

1. Collections of engorged female mosquitoes are made and the insects transferred alive to the laboratory.
2. The mosquitoes are killed and identification is made as to species.

3. With a pair of forceps a mosquito is secured near the anterior end of the abdomen and the lower part of the abdomen is squeezed so that the blood will spread and be absorbed over a small piece of filter paper.

4. Pertinent data as to collecting place, resting site, date, time, name of collector, conditions under which collections were made, nearest likely blood source, possible blood sources in the area, prevalence of mosquitoes, etc., are recorded and the specimen of blood on the filter paper is stored in a cool, dry place until the precipitin tests can be made.

5. The blood is extracted by soaking the filter paper in physiological salt solution for an hour or more. If the process takes over two hours, extraction should be done in an icebox.

6. The supernatant fluid from the above is then carefully layered onto human antiserum. (See medicolegal tests for blood.) Antisera other than that of man may be used depending upon the likely occurring hosts of the mosquitoes in the area under observation.

Poisonous Snakes and Lizards*

Snakes

Snakes belong to the class Reptilia and the order Squamata, suborder Ophidia. The two families to which poisonous snakes belong are the Colubridae (colubrine snakes) and Viperidae (viperine snakes).

Although the toxicity of the venom and the amount normally present are matters of great importance in estimating the lethal powers of species of poisonous snakes, the principal feature to be considered is the ability of the fangs to introduce venom into the tissues of the animal bitten. For example, in the Opisthoglypha there are fangs attached to the maxilla but these are placed posteriorly to the solid teeth in front, so that, since the venom cannot be inoculated, these snakes are from a practical point of view nonpoisonous. Then, too, snakes in which the fangs are so situated have only a small poison gland and their venom is of low toxicity. In dangerous snakes the poison fangs are placed anteriorly, attached to the maxilla, which, in the poisonous Colubridae, is long and lies horizontal and, in the Viperidae, is short and lies vertical.

The nonvenomous snakes are in the Aglypha series and have solid teeth. There has been a question as to toxicity of the saliva of some aglyphs, but this is probably an allergic manifestation. With the Protereoglypha, where the dangerous snakes belong, there are grooved or canalized poison fangs, attached anteriorly to the maxilla.

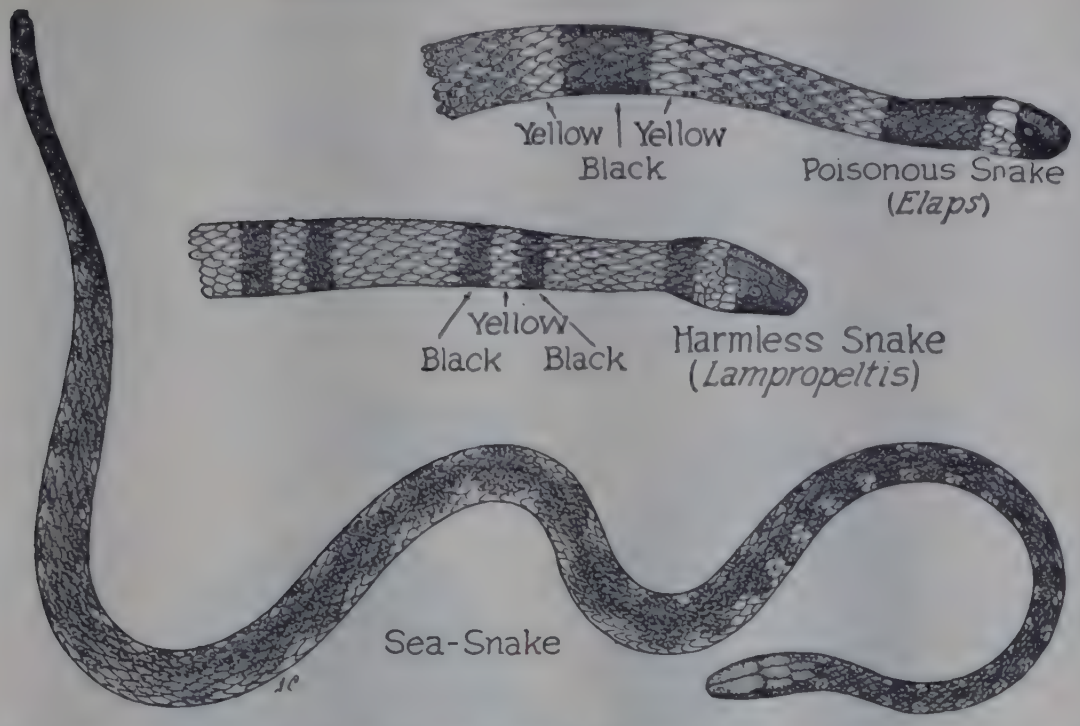
Colubridae. The three series noted above belong to this very large family. The poisonous species belong either to the Hydrophinae (sea snakes), which have an eel-shaped tail and a rather flattened body, or to the Elapinae (land snakes), which have a round tail. As a rule, sea snakes live in salt water near the shore, but such snakes have been reported from fresh water lakes in the Philippines. They are of importance in the Tropics and are a source of danger to fishermen. While their venom is extremely toxic, and their fangs situated anteriorly, the danger from them is minimized by their small heads and relatively inefficient bite. The Elapinae have short, strong fangs anteriorly located, and behind them small grooved (not canalized) teeth. The poison gland, which is the homologue of the parotid, has a duct located in the upper lip and terminating in a papilla. The poison duct does not enter the fang lumen but empties into muscular folds which surround the base of the fang, hence breaking off of a fang does not necessarily injure the duct. There is a succession of teeth in snakes, so that a new fang grows out if the original one is extracted.

Many of our harmless snakes such as the garter snake and black snake belong to the Colubridae.

The cobras belong to the subfamily Elapinae and are best known by a necklike expansion or hood. The only poisonous colubrine snakes in the United States are the bead snake (*Micrurus fulvius*), often called the Florida coral snake, and the Sonoran coral (*Micruroides curyxanthus*). Both of these snakes were formerly included in the genus *Elaps*.

The bead snake is black with about 17 broad crimson bands bordered with yellow. Although small, it is very venomous. The upper jaw has anteriorly grooved fangs, which

*Revised by Paul W. Clough, M.D.



The poisonous coral snakes of the United States, *Micrurus fulvius* and *Micruroides euryxanthus* (formerly *Elaps*), have transverse rings of black, vermillion and yellow. Differentiating these snakes from harmless ones which resemble them there are black rings bordered by two yellow ones, while with the harmless snakes a yellow ring is bordered by two black ones. The sea snake (*Enhydrina* species) has a rudderlike tail which is here shown twisted to one side.

appendages are not present in the nonpoisonous coral snakes, these latter having teeth in the upper jaw so that the wound shows four rows of punctures instead of two rows and one larger puncture on each side to mark the entrance of the fangs.

In Asia there are many important poisonous colubrine snakes, the cobra (*Naja tripudians*), the King cobra (*Naja bungarus*) and the kraits (*Bungarus candidus*).

All of the Australian poisonous snakes are colubrines.

Snakes of the United States (Stiles)

(*A*) Pupil of eye vertical; pit present; single row of ventral scales posterior to the vent; adults with head more or less triangular, constriction behind head more or less prominent ..... Pit vipers (all poisonous)

(*B*) Pupil of eye circular; pit absent; double row of ventral scales posterior to the vent

(*a*) Color: yellow, black, yellow, red, in bands ..... Coral snake (poisonous)

(*b*) Color: black, yellow, black, red, in bands ..... False corals (not poisonous)

(*c*) Color: all others ..... Not poisonous

Viperidae. The viperine snakes are characterized by a broad head, narrow neck, short and stumpy tail, and a short upper jaw which, with the fangs, is directed obliquely backward. The rattlesnake (*Crotalus*), the copperhead (*Ancistrodon contortrix*), and the water moccasin (*A. piscivorus*) are widely distributed in the United States.

There are many harmless snakes which more or less resemble these "pit vipers," as the rattlers, moccasins, and copperheads are called. This term refers to a deep hole or pit found on the side of the head between the nostril and the eye. It is a blind sac. The much dreaded "fer-de-lance" (*Bothrops lanceolata*) is a crotaline snake.

Some divide the Viperidae into the Crotalinae, which possess the pit, and the Viperinae which do not have this structure. Russell's viper (*Daboia russelli*) is the best known of the Viperinae and is one of the most important poisonous snakes of India.

The poison fangs are grooved or perforated and connected with the poison glands

which resemble salivary glands and may be almost an inch in length in large snakes. The tongue is slender and forked and is a tactile organ.

The jaws are remarkable for their great extensibility, not only vertically, but laterally, permitted by the ligamentous connections of the two halves of the mandible or lower jaw.

As the fangs are directed backward it is necessary for the snake when striking to open the jaws widely and bend back the neck. The fangs are then brought forward and erected by the sphenopterygoid muscles. Barnes has pointed out that in vipers the venom gland lies between the fibers of the maxillary and sphenopterygoid muscles and



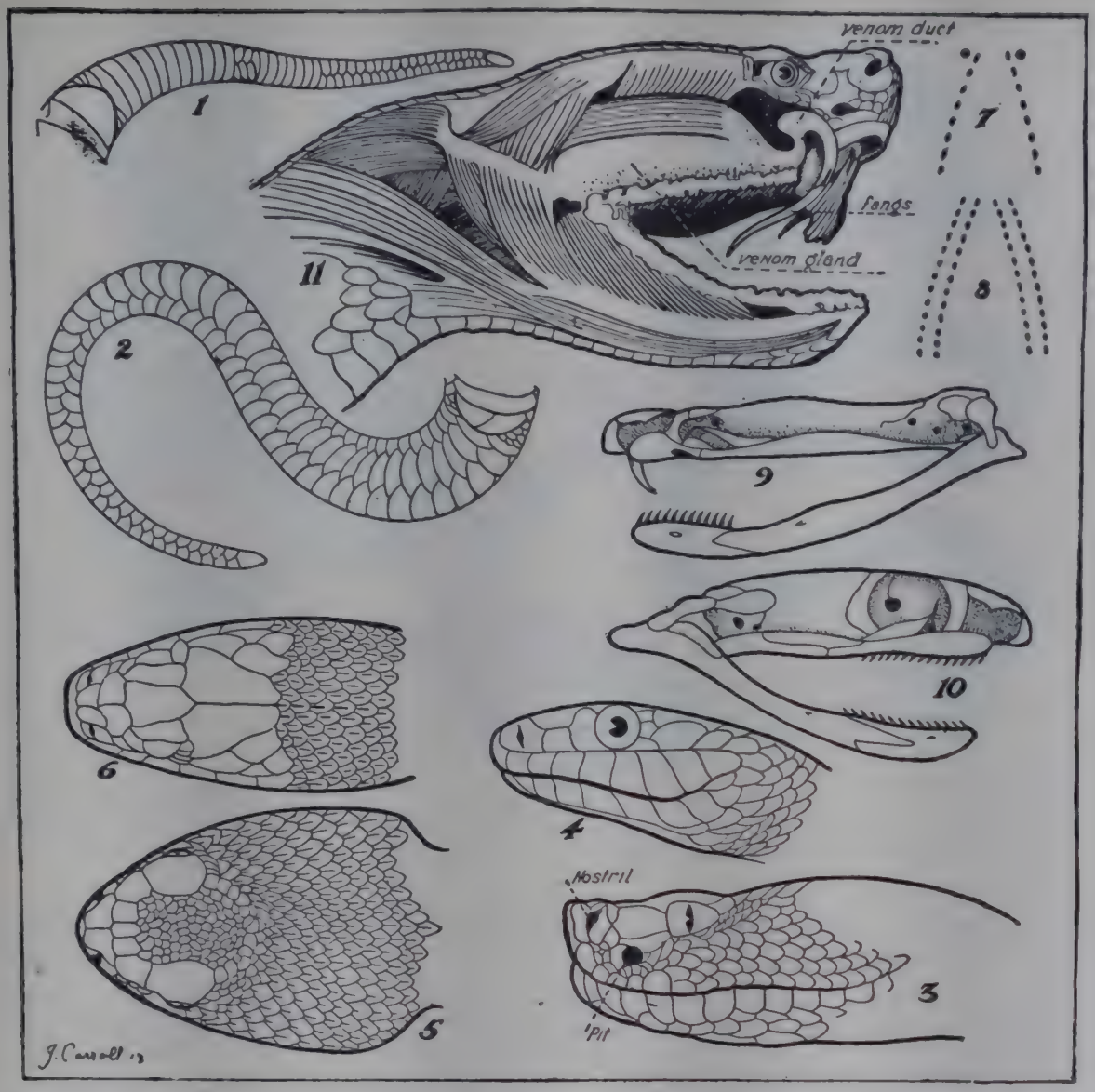
Daboia russelli. (After Mense.)

that the contraction of the latter in erecting the fang would also squeeze the venom gland and eject the venom. The snake bite is a combination of bite and blow. The functional fangs of colubrine snakes, however, are not mobile.

In addition to the possession of the pit these vipers have a more or less triangular head and in particular a single row of large scales on the under surface posterior to the vent (anus), while the harmless snakes show an elongated oval head and two rows of large ventral scales posterior to the vent.

Snake Venom. In examining the wound made by a snake the two punctures of the fangs indicate the bite of a poisonous snake. If these fang-puncture points are far apart it shows that a large snake, and probably one capable of injecting a greater amount of venom, has given the bite.

When a snake strikes the fangs move from the horizontal to the erect position, the mouth being widely open. When the fangs enter the jaws close and pressure is exerted on the poison glands so that the venom pours out.

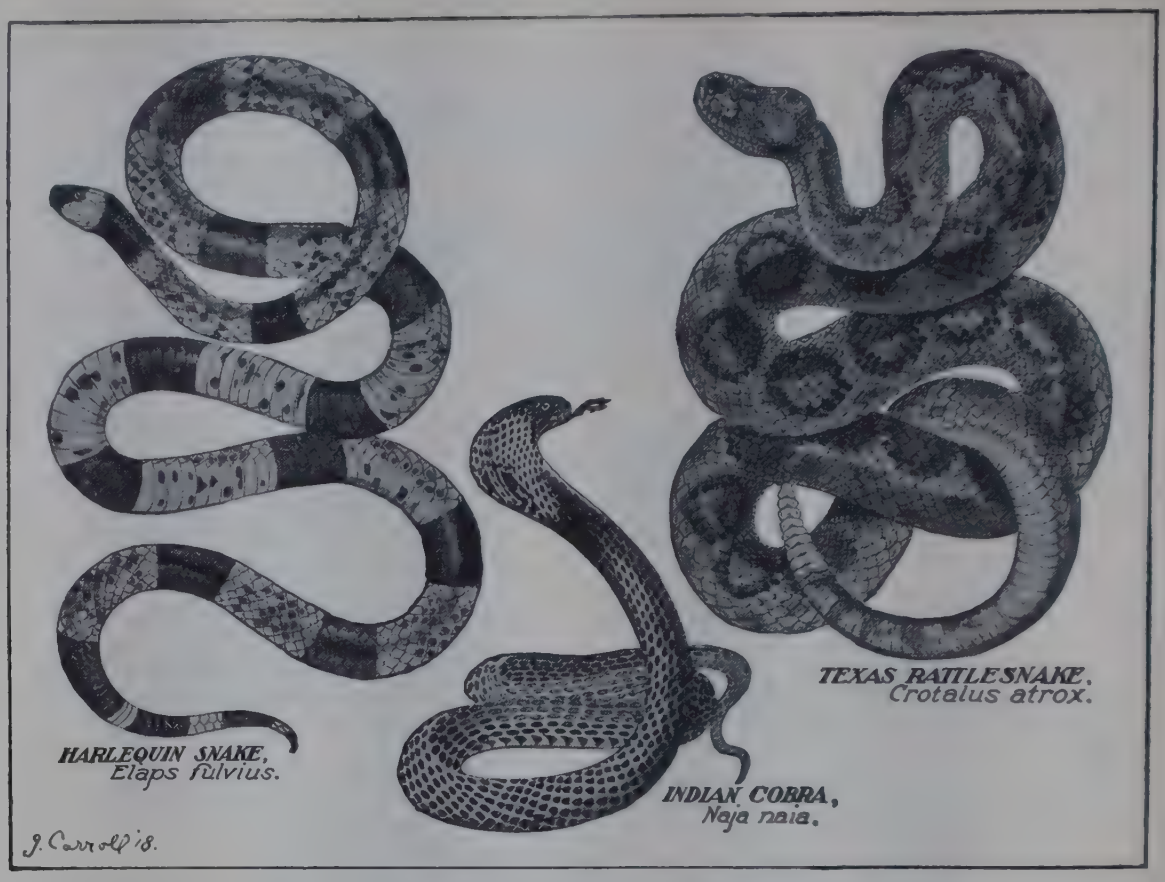


(1) Single row of scales posterior to vent (poisonous snake—water moccasin). (2) Double row of scales of harmless snake (*Natrix*). (3) Side view of head of pit viper. (4) Side view of head of harmless snake. (5) Dorsal view of pit viper. (6) Dorsal view of harmless snake. (7, 9) Bite puncture and skull of *Micrurus*. (8, 10) Same of harmless snake. (11) Poison apparatus of rattlesnake.

The idea that a snake exhausts its venom when striking is not true. Colubrine snakes may bite shortly after the first attack, and inject each time a lethal dose of venom. Fresh venom varies from an almost colorless fluid to one with a brownish or greenish color. It is viscid and quickly decomposes from the varied bacterial flora it contains. A number of years ago the injection of rattlesnake venom was used in the treatment of epilepsy but dangerous and even fatal reactions resulted from the pathogenic anaerobes at times present in the venom of snakes. Dried venom is quite stable in the dark, and retains its toxicity for years.

The amount of venom varies with the size and condition of the snake, an adult cobra yielding about 1 ml. Acton and Knowles give the following table expressed in milligrams of desiccated venom.

Common cobra (mean yield)	317.0	mg.
Common krait (mean yield)	8.17	mg.
Banded krait (mean yield)	64.4	mg.
Russell's viper (mean yield)	108.0	mg.



Important poisonous snakes. (*Elaps fulvius* is now included in the genus *Micrurus*.)

They estimate the minimum lethal dose for man as 15 mg. with cobra venom and 42 mg. with the venom of Russell's viper (*Daboia*). The venom of the kraits is more potent, that of the very common Indian krait, *Bungarus candidus,* being given as 1 mg.

The cobra, after having bitten, remains attached for a short time while the *Daboia* strikes with the greatest rapidity and immediately releases itself.

Cobra and krait bites (colubrine snakes) produce more or less similar symptoms such as paralysis of articulation with nausea and vomiting and later paralysis of the respiratory apparatus. There is only an insignificant reaction at the point of bite.

The venom is mainly neurotoxic, causing death by paralysis of cardiac and respiratory centers. Cobra venom is also very hemolytic. This hemolysin is activated by the normal complement of the serum of the animal poisoned, the hemolysin as contained in the venom not being toxic when alone. Lecithin also has the property of activating the hemolytic substance in venom.

In rattlesnake bites (viperine snakes) there is marked pain at the site of the wound with much swelling and hemorrhagic infiltration. The swelling and petechial mottling spread up the limb from the point of entrance of the venom. Cold sweats, nausea, cardiac depression, and syncope are common. An exception to this general rule is *Crotalus terrificus,* whose venom is strongly neurotoxic, affecting vision and respiration. The local effects are slight.

Rattlesnake venom is active chiefly on account of its hemorrhagin, or rather endotheliolysin, which destroys the endothelial lining of blood vessels.

The hemolytic (hemotoxic) effects of the venom of the West Indian and Central American vipers are most marked—hemorrhages from the conjunctivae and stomach occurring along with reflex vomiting. There is marked damage to the blood vessel walls, death occurring in coma in about eight hours in the absence of antivenin. Even with such treatment transfusion may be necessary. Of the American pit vipers, the rattlesnake venom is the most toxic and that of the water moccasin least so, but the necrotizing power of the latter is more marked.

Venoms may also contain proteolytic ferments which may account for the softening of muscles in snake-bite cases. The toxic effect of the venom takes place without an appreciable incubation period, hence different from true toxins.

The most venomous snakes seem to be the sea snakes (*Enhydrina*). This venom is almost entirely neurotoxic.

The tiger snake of Australia is almost equally venomous and the krait (*B. candidus*) next. The rattlesnake is about one-fifth as venomous as the krait.

Certain venoms greatly increase the coagulability of the blood so that intravascular thromboses may occur. It is chiefly with the venoms of *Daboia* and *Bungarus* that such thromboses are likely to occur and this accounts for the almost instantaneous death which at times results from bites of such snakes, when the toxin is injected directly into a vein.

Treatment of Snake-bite Poisoning. The nonspecific treatment of snake-bite poisoning which has usually been recommended is: (1) Application of a tight ligature above the site of the bite for 20 to 30 minutes. The ligature, which should preferably be a rubber band, is to be applied about a single-bone extremity, not about one with two supporting bones. (2) It is recommended that a piece of rubber gauze be cut and placed on the site of the bite. Suction by mouth should then be kept up steadily for at least one-half hour if no antivenin is available. If antivenin is at hand, it should be administered at a distance from the site of the bite, and the suction continued. Incision increases the chance of secondary infection.

Bannermann has shown that a dog bitten by a cobra cannot be saved by free incision and the rubbing in of permanganate crystals. It may, however, be saved by the immediate injection of 10 ml. of a 5 per cent solution of permanganate, but not if two minutes have elapsed. Bites from the *Daboia* are fatal, however the permanganate be applied. Bannermann, therefore, does not consider the permanganate treatment of any practical value. Rogers thinks that Bannermann's experiments with dogs do not give a true idea of the value of permanganate because he has had success in experimenting with cats and because he believes it has saved human lives. Chromic acid injections (1 per cent) have also been recommended. Acton and Knowles consider potassium permanganate as unreliable and recommend subcutaneous injections of a 5 per cent solution of gold chloride. These local injections may be helpful if used before the venom has been absorbed but they have no effect on venom taken up by the circulation. Intravenous injection of permanganate is not only without effect but is dangerous. Amaral states that the ligature will not prevent the venom from spreading and may accentuate the proteolytic and cytolytic action. In his opinion permanganate solutions in active concentrations have a deleterious action on tissues. *The use of potassium permanganate is now generally regarded as contraindicated.*

Internally alcohol does not seem to be of any value; in fact, many of the deaths have been attributed to excessive ingestion of whiskey. Strychnine in large, almost poisonous, doses was highly recommended in Australia, but the statistics seem to make the value of this remedy doubtful.

In an article on snake bite, N. Hamilton Fairley (1934) states that early free excision combined with mechanical suction is the only method of local treatment likely to be successful in body bites. Immediate application of a ligature and free excision (3 by 3 cm.), down to the muscles, was the only effective local treatment in sheep bitten by tiger snakes. He refers to the work of Crimmins (1927), advocating ligature and incision combined

with suction by a breast pump, and that of Jackson and Githens (1931), in which incision of the wound bite, combined with suction by a Bier's apparatus and irrigation with saline, is recommended. Fairley regards these methods as useful accessories to ligature, incision, and excision. As regards the use of permanganate, Raymond Ditmars (Cecil's Medicine) states "Nothing could be more foreign to the treatment of snake bite than such practice." For local treatment he advocates deep incision and forced suction. *Cauterization should never be used.*

Allen (1939) thinks the earliest possible excision of a large area of tissue may be beneficial. In still more desperate cases amputation is a more positive remedy. He thinks that the tourniquet may be serviceable, with or without refrigeration, as a preliminary to delayed amputation. He believes the use of an *occlusive* tourniquet is harmful, and does not recommend incisions.

Herbert Clark (1942), who has had more than 30 years' experience in Central America, emphasizes the necessity for *immediate* treatment. The snake should be killed for identification. He advocates the use of a tourniquet sufficiently tight to stop venous but not arterial flow. This should be loosened for a few seconds every 10 minutes. Extraction of the venom by suction should be started at once. Suction may be applied by the mouth over a thin sheet of rubber laid over the bite. This should be continued vigorously for five minutes, the site should then be washed, antivenin given, and suction repeated. Incisions are not advised as they increase the raw surfaces and hasten absorption. Antivenin should be given subcutaneously, and the tourniquet should be kept in position for an hour after the administration. Alcohol is contraindicated, and potassium permanganate cannot reach the venom beneath the skin.

Antivenins. The active agents of snake venoms may be either of the nature of hemorrhagins, neurotoxins, or fibrin ferments. In colubrine snakes the neurotoxin vastly predominates whereas with the viperines it is the hemorrhagin. Certain Australian snakes contain all three toxins in about equal proportion, whereas with the rattlesnakes of America it is almost entirely the hemorrhagin which causes the poisoning. The *Micrurus* (*Elaps*) of Florida is a colubrine snake and its venom is neurotoxic in nature.

The cause of death in colubrine snake bites is chiefly from paralysis of the respiratory centers whereas with the pit vipers it is chiefly from hemorrhages in the vital organs. Antitoxins have been prepared against both viperine and colubrine venoms and these are specific; thus a colubrine antivenin will not be of value against a viperine bite. Antivenins should be administered either intravenously or intramuscularly. The amounts recommended for injection to neutralize a fatal dose of snake poison vary from 100 to 300 ml. of the antivenin serum. There is no accurate method of standardization.

In Central America antibothropic serum can be given in all cases with a good chance of effectiveness, since 80 to 85 per cent of the bites are due to species of *Bothrops*.

When Calmette (1894) first produced antivenin the idea prevailed that it was useful for any snake venom, a view soon found to be untenable. There are now institutes in many parts of the world where antivenins are made to combat the local venoms; thus in the U. S., there is the Antivenine Institute of America which produces an antivenin for rattlesnake, copperhead, and water-moccasin venom. These venoms are chiefly hemorrhagic. Previously, the toxicity of some venoms made the immunization of horses precarious, but methods of detoxication are now being used which are more successful. Both monovalent and polyvalent sera are produced. Often, when it is impossible to determine the species of the offending snake, a polyvalent serum is indicated. Antivenins are given either intramuscularly or intravenously. With highly poisonous venoms intravenous therapy is indicated. Fairley emphasizes that dosage is in inverse propor-

tion to body weight, so that children may require several times the amount of serum sufficient for a heavier adult. This is because of the natural neutralizing power of the blood stream. A large individual, having more blood partially to neutralize venom than a smaller person or a child, requires less antivenin. Owing to varying strengths of antivenins (concentration methods) one should depend for dosage on the instructions accompanying the product. Besides the local and specific treatment for snake bite one should put the patient at rest physically and mentally, as psychic shock is an important matter with some snake-bite patients. Fairley also recommends black coffee or caffein. Strychnine and alcohol, and in particular morphine, should be avoided.

Lizards

Lizards are nonpoisonous with the exception of the two Gila monsters (*Heloderma suspectum* and *H. horridum*). The first is found in Arizona and New Mexico (Gila river valley), the second in southwestern Mexico. They are about two feet long, heavily built, and covered with small tubercles. The name monster is most applicable. The poison fangs are in the lower jaw, and the bite of these apparently sluggish creatures may cause death. When aroused, they are very vicious and it is as difficult to open the closed jaws as in the case of a bulldog. They deposit parchment-like eggs in the sand of the desert.

Poisonous Fish and Coelenterates*

Poisonous Fish

Fish Poisonous as Food. Illness produced by eating decomposed fish, whether in the natural state or canned, belongs to the general problem of food poisoning. There are, however, certain fish whose meat is poisonous when eaten in a perfectly fresh state.

This may be connected with certain epidemic diseases among fish ordinarily utilized as good food. Various bacterial organisms have been isolated from such fish, and the poisonous effects have been attributed to various ptomaines elaborated by these toxicogenic organisms. Most of the organisms isolated from diseased fish have belonged to the colon or proteus groups. Cases have been reported of botulism-like poisoning arising from the eating of insufficiently salted fish. These cases were probably due to the development of a soluble toxin by *Cl. botulinum,* as such fish when cooked lost their toxicity. The toxin of *Cl. botulinum* is destroyed by heat, whereas that due to Salmonella, or ordinary food-poisoning organisms, withstands ordinary cooking temperatures. This fish poisoning by bacterial products is designated *ichthyotoxismus.*

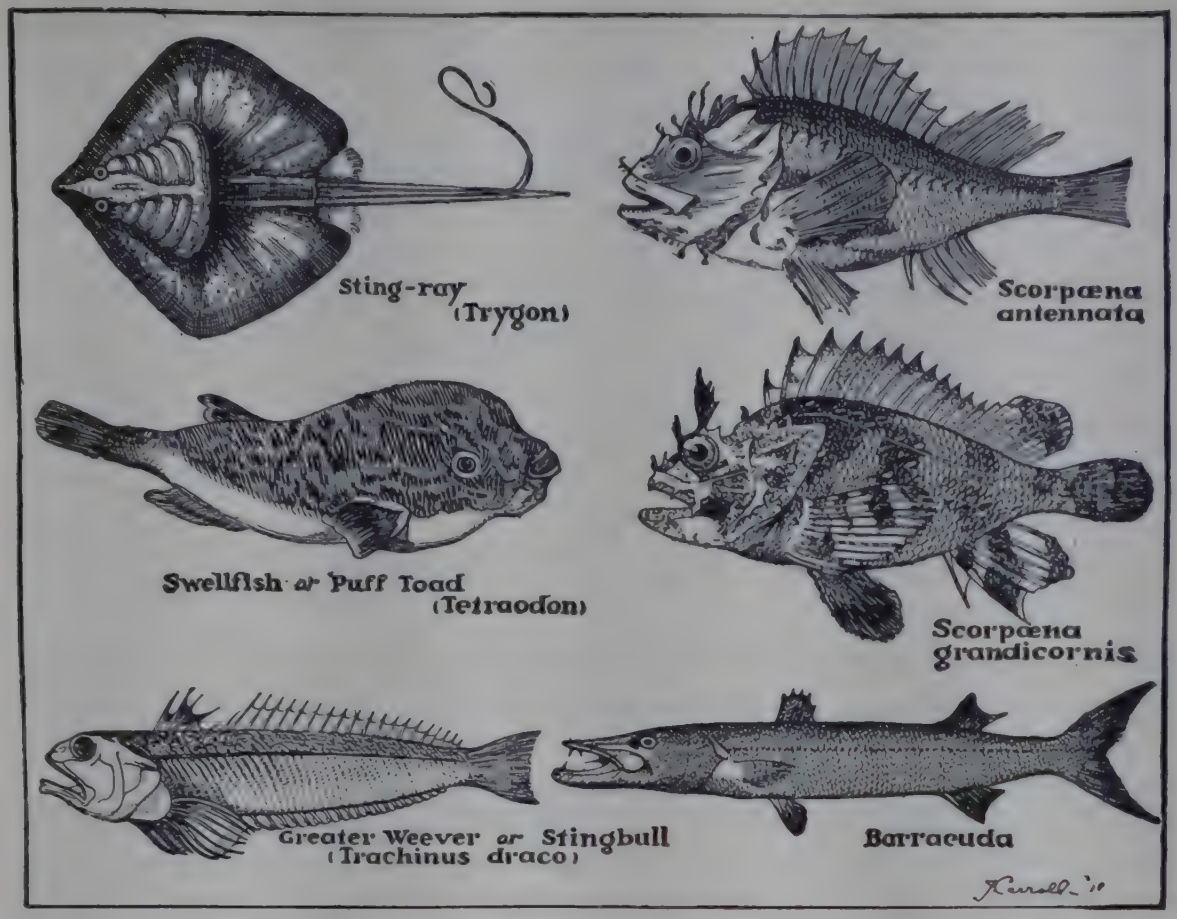
There are certain fish whose meat is poisonous when there is no question of decomposition or disease in the fish. The best known instances are certain species of the genus *Tetrodon.* The illness produced by the eating of this fish is usually termed *fuguismus,* the Japanese designating such fish by the term "fugu." The poisonous principles seem to exist chiefly in the ovaries and testes, the eating of even one roe of such fish bringing on serious illness in a few minutes or possibly death in a few hours. It has been stated that after careful removal of all genital and alimentary-tract organs these fish may be eaten without harm. The poisonous principle has a physiologic action somewhat like curare, and is thermostable. Such fish have been used as agents in committing suicide. Boesoirie (1940) has reported from the Netherlands Indies two deaths among nine individuals exposed, following the ingestion of *Tetrodon argenteus.*

The porcupine fish or Diodontidae are considered as poisonous. These fish together with the Tetraodontidae, or broad-nosed puffers, are unsightly in appearance. Among seamen they are generally designated "puff toads" since they become distended with air as they are drawn out of the water. It is well recognized that certain of these fish which may fail to cause poisoning at one time may do so at another time and it is particularly noted that poisoning effects occur at the time of spawning.

In the Tropics fish which may ordinarily be safe as food become poisonous as a result of feeding on certain poisonous medusae and corals.

This is probably true of the barracuda, which is eaten with impunity at most times. Yet undoubted cases of poisoning with this fish have occurred. It has also been suggested that the barracuda may be poisonous at certain times in its life, for example, during spawning, or that it is subject to a more rapid decomposition at such times.

*Revised by Paul W. Clough, M.D.



Poisonous fish.

To test reports of such poisoning, Bloedorn and Hakansson (1926) reported the eating of barracuda with safety on several occasions. This fish was one of those most commonly caught in the West Indian waters surrounding Puerto Rico. The fish, eaten on several occasions, varied from 8 to 15 pounds and one weighed 26 pounds. One of the warnings from local fishermen was that very large barracudas were not safe. Fish poisoning in the Tropics from barracuda or other edible fish is probably most often due to commencing putrefaction.

There are certain species of the herring family which have a bad reputation. Among these are two species of *Meletta*. In New Caledonia, *M. venenosa* causes painful cramps of the body with dyspnea, cyanosis, cold sweats, and dilated pupils and at times death. *M. theissa* of the West Indies is also a very poisonous fish.

Herre describes in the Philippine Journal of Science for October, 1924, 60 species of poisonous and worthless fishes, of the order Plectognathi.

Macht (1941) investigated the toxic effects of fresh muscle juices of 65 different varieties of fish in the United States by injections into mice and by observing the effect on the growth of *Lupinus albus* seedlings. All the fish they found to be toxic are regarded as inedible except the catfish and the eel.

According to Barbour (1941) eating the flesh of certain poisonous species of fish causes a disease known in the Caribbean area as Ciguatera, the symptoms of which vary with the species and the susceptibility of the individual. There are two clinical types. In the more frequent and benign there is a gastroenteritis with headache, epigastric pain, cramps, tenesmus, nausea, syncope, and erythrism, and death may occur. In the algid form symptoms resulting from injury to the nervous system come on rapidly, with alternating periods of paralysis and convulsions, cyanosis, coma, and death. Many cases of the algid type have been reported from New Caledonia after eating a local herring, *Clupea venenosa*, and a toad fish, *Tetrodon maculatum*.

Toxicity appears in a sporadic manner in one genus and not in another of the same

family, or in one species and not in another of the same genus. There do not appear to be marked individual differences in the same species under the same conditions as to age, time of year and sexual maturity. The development of the toxin is believed to be the result of the exaggeration of a normal metabolic function and not a pathologic phenomenon. The toxicity is most marked when sexual processes are most active, that is, during the breeding season. For example, the eggs of our pickerel are venomous.

Fish Which Poison by Their Sting or Bite. According to Barbour (1941) many spiny fishes secrete an irritant poisonous slime which may be generally distributed over the surface of the body, or more frequently is located in glands situated at the base of highly specialized spines which serve to inject the poison. The sting rays, *Trygon* or *Dasybatis,* are typical among Elasmobranchs. *Scorpaena, Chilomycteris,* and the freshwater cat fishes such as *Ameiurus* are examples among the Teleosts. In some of these the poison is very virulent, in others only slightly irritant.

Fish of the genus *Muraena* have specialized teeth associated with a poison sac which secretes a venom which is introduced into the wound made by the bite.

There are various rays which are well known all over the world as capable of inflicting wounds. In the sting rays (Trygonidae) the tail is armed on the upper side with a barbed spine which in some species is connected with a poison apparatus. Some of these sting rays when wounding a person who may step on them while wading in the water may at the same time inoculate tetanus bacilli which are particularly dangerous because of the character of the deep-punctured wounds. In the electric rays (Torpedinidae) the dorsal surface is electrically positive and the ventral one negative. To receive a shock one must communicate with the *Torpedo* species at two distinct points. Some of these electric rays are capable of temporarily paralyzing the arm of a man.

Two of the best known poisonous fish are *Trachinus draco* and *Scorpaena scropha.* The flesh of these fish is wholesome as food. *T. draco* is like a trout in appearance and has blue and brown stripes. It has a grooved spine passing through each of its gill covers which is connected with a poison gland. There is also a poison apparatus connected with the dorsal fin. *S. scropha* is an ugly, red fish with large head and prominent fins. The French fishermen call it "le diable." The poison apparatus is connected with the first three rays of the dorsal fin.

Persons in bathing who strike against these fins are more apt to be wounded than the fishermen who handle the fish with caution. After being wounded a person experiences stabbing pains in the affected part. A sensation of suffocation follows and the victim may become delirious. At times collapse and death result. At the site of the wound at first there is an erythematous area which later becomes black and may become gangrenous. As the poison rapidly enters the general circulation, treatment similar to the local treatment of a snake bite is called for. These fish seem to be more dangerous during the spawning period.

S. plumieri, the lion or scorpion fish, has been reported by Bayley (1940) to frequent the shoals of the fine bathing beaches in Barbados, where it hides in weed-covered crevices of the coral. Bathers may be injured by striking its sharp spines or by stepping on the fish. The poison injected by the spines often gives rise to very severe symptoms—violent pain comparable to renal colic, sweating, pallor, dyspnea, tachycardia, vomiting, diarrhea, and often later a rash.

The only two important animal parasites with which the eating of fish is connected are: (1) *Diphyllobothrium latum* and (2) *Clonorchis sinensis*. The broad Russian tapeworm is a rather common parasite of man in the Baltic provinces and comes from eating insufficiently salted pike and other fish infected with this larval tapeworm. The liver-fluke disease of China and Japan is caused by eating various raw or insufficiently cooked fresh-water fish. These fish are the secondary intermediate hosts, the primary ones being mollusks. A very small fluke of Japan, *Metagonimus yokogawai,* is transmitted by the ingestion of certain goldfish.

Mollusks and Disease

The importance of disease transmission by snails has been taken up under the helminthic infections caused by flukes.

Oysters. There are more than 100 species of true oysters (*Ostrea*), exclusive of various allied forms, such as the pearl oyster or the window-pane oyster. Oysters belong to the class Lamellibranchia and the two most important edible species are *O. edulis,* the European oyster, and *O. virginica,* the Americo-Canadian one. The question often arises as to the edibility of oysters which are green in color. This color is considered desirable in certain European countries, and is produced in France by feeding the oysters a diatom, *Navicula ostrearia.* The green pigment is present in the gills and palps. Such greening may be natural. Sometimes there is a green color in the body of the oyster due to copper.

It is probable that the only ground for considering a sound oyster as capable of causing food poisoning is from its effect on individuals with an idiosyncrasy to shellfish—and such idiosyncrasy seems not uncommon.

Typhoid Fever and Oysters. There have been numerous outbreaks of typhoid fever in both the United States and England for which the eating of contaminated oysters was responsible. Oysters may be contaminated when growing in sewage-contaminated beds, but in the studies of typhoid infection from eating raw oysters the blame has been placed on polluted water used for floating oysters. If the water in which the oysters are placed for storage (floating) is pure, such a process makes the oyster safer. At present, floating in chlorinated sea water is of particular sanitary advantage. Outbreaks of typhoid fever, as for instance that of the New York epidemic of 1924–1925, when more than 100 deaths from typhoid fever were recorded, have led to official regulation. In uncontaminated salt water, studies have shown that the oyster gets rid of the typhoid organism in from two to three weeks.

Mussels. These also belong to the class Lamellibranchia. Mussels are widely distributed, and in Europe form an important article of food (the edible mussel, *Mytilus edulis*). They seem to thrive better in saline waters which have a somewhat lesser salt content than sea water. Fresh-water mussels have an economic value in the use of their shells for button-making. Besides ill effects related to idiosyncrasy, we may have poisoning leading to paralysis or even death. In California (1927), there were reported 102 cases of mussel poisoning with six deaths. The symptoms developed in 10 to 20 minutes after ingestion. The toxin seems to be thermostable and is absent except during the spawning season (June through September).

Poisonous Coelenterates

The phylum Coelenterata includes animals of very simple structure, only the sponges and protozoa having a more lowly type. It is customary to distinguish two morphologic types of coelenterates, the polyp and the medusa.

Polyps. The best example of a polyp is a sea anemone.

Quite interesting in the study of immunity is the constant association of an anemone with certain hermit crabs. The anemone covers the soft tail-end of the crab, thus protecting the crab from attacks by its enemies. The mouths of the two animals are in close juxtaposition so that the food of the crab is shared with the anemone. This crab acquires an immunity to the poison of the anemone, probably as the result of frequent ingestion of fragments of anemone. Other crabs are very sensitive to the anemone poison, suffering paralysis and death. The poison of certain anemones may even harm other anemones.

A condition known as "la maladie des plongeurs" occurs among the sponge fishermen of the Mediterranean. This is due to stinging by anemones and is characterized by marked itching, burning, and erythema. In some cases the skin of the affected area becomes necrotic and sloughs off leaving an ulcer.

Levin and Behrman (1941) in the West Indies have described a dermatitis caused by the coral polyp of the genus *Actinia*. Species of the genus *Alstinion* and *Hellenopolypus* may cause nausea and vomiting in addition to local lesions. Applications of vinegar and olive oil have been recommended for the local symptoms.

Jellyfish (Medusa). This umbrella-like coelenterate has tentacles which hang down from the margin of the organism.

As a rule jellyfish are harmless but certain species produce unpleasant or even serious effects by their sting. Lesions following contact with unspecified "jellyfish" have been reported by Allen (1920) and Stewart (1922). The local rash in Allen's case was followed by profuse weeping eczema, by aphonia, and by laryngitis lasting for four weeks. Aoki (1922, 1923) portrays the severe effects, besides urticaria, of stinging by *Olindioides formosa*—shock, acute cardiac distress, dyspnea, muscle pains, and as a possible sequela emaciation.

In the Mediterranean a jellyfish *Rhizostoma pulini* produces edema and urticarial eruptions as the result of its sting. In many parts of the Tropics jellyfish are found which give rise to quite serious symptoms. In the Philippines there are certain species of jellyfish which cause serious illness, although as a rule one experiences no discomfort from coming in contact with many other species while swimming in the waters of that part of the world.

According to Light, the species of *Dactylometra*, called "fosforo" by the natives, is the most dangerous one there encountered. It has long ribbon-like oral lappets and 24 slender white, marginal tentacles. In this the sting is inflicted by nematocyst batteries in the four long, ribbon-like oral palps. *Lobonema*, called by the natives "lanterna," is of large size, white or white and purple in color, and stings by the long filaments which arise from the mouth arms. Stitt has treated a number of patients stung by jellyfish in the Philippines; the symptoms ranged from a mild erythema to marked congestion of the respiratory tract and other general symptoms.

Old has described these symptoms very accurately and notes the following:

The symptoms appear in from 10 to 60 minutes with marked hysterical manifestations, incessant cough, and coryzal signs. Light believes that the cases described by Old were due to stinging by *Dactylometra.*

Wade describes his own experience with a jellyfish sting while swimming in Manila Bay. The tentacles became wrapped about the upper arm and stinging was instantaneous as the tentacles did not cling. The poison did not reach the conjunctiva or other mucous membranes. There was at once a sensation of burning in the area of contact, but it was 15 minutes before other symptoms appeared. There was pain in the loins and also in the scrotum. This was followed by a curious restlessness and weakness, then a sense of constriction in the throat, with chest discomfort and then coryza and lacrimation. The symptoms abated and within an hour there only remained weakness and soreness of the bronchi. A vesicular dermatitis appeared on the arm and the traces of the sting had not disappeared after two or three weeks. Other cases have been reported associated with feeble heart action and semiconscious states. There is always to be considered the possibility of one's drowning when in the emotional or semiconscious state. Wade describes a death in a robust Filipino who was stung on the leg. His companions were only a few yards away, but by the time they had reached him he had collapsed and was gasping and livid, and was dead a few moments later. It was at first thought he had been bitten by a sea snake but there was no mark on the leg, except conspicuous purplish discoloration. On autopsy, status lymphaticus with persistent thymus, acute congestion of the viscera, and edema of the lungs were demonstrated.

The Portuguese man-of-war (*Physalia*) has long locomotive tentacles which stretch out 30 to 50 feet as the animal is blown along by its pearly purple-crested bladder-like float or sail. The thread cells are capable of inflicting rather painful stings when handled without a knowledge of the effect of coming in contact with these thread cells.

Along the coast of eastern Florida great swarms of a small, very dark brown *Medusa* occasionally appear near the shore (*Linuche unguiculata,* formerly better known as *Linerges*). They are so abundant that very severe stinging occasionally occurs. The symptoms are similar to those described above.

PART IV

Clinical and Pathologic Examinations of the Various Body Fluids and Organs

By Paul W. Clough, M.D.

Chapters 30–43
By Paul W. Clough, M.D.

Chapter 44
By M. Pijoan, Lieutenant Commander (MC) USNR

Diagnosis of Infections of the Ocular Region

Collection of Material. The skin about the inner canthus should be cleansed with sterile salt solution. A drop of exudate may be extruded by gentle pressure on the lids, or the lower lid may be everted. A sterile, small, cotton toothpick swab may be used, first for inoculating media and also for making smears. If exudate is abundant a rubber-bulb capillary pipet, the tip of which has been flame-polished, is convenient for securing a few drops of material. If exudate is scanty a few drops of sterile broth may be instilled into the conjunctival sac, and a little removed for culture. Blood agar plates should be used, or suitable special media if some specific organism is suspected.

Bacterial Infections. The xerosis bacillus and the *Micrococcus* (*Staphylococcus*) *albus* may be considered normal findings in the conjunctival sac, although the latter may be the cause of a phlyctenular conjunctivitis. Streptococci may cause inflammation of the nasal duct or a pseudomembranous conjunctivitis.

The diphtheria bacillus, which morphologically resembles the xerosis bacillus, may cause a pseudomembranous conjunctivitis. Great caution should be used in ascribing pathologic significance to the xerosis bacillus.

Pneumococci are fairly common as a cause of conjunctivitis, dacrocystitis, and even panophthalmitis after trauma or operations, especially for cataract. They may also cause serpiginous ulcerations of the cornea, particularly if the epithelium has been injured. These infections are serious and require prompt treatment.

Hemophilus conjunctivitidis (Koch-Weeks bacillus) causes a very acute and highly contagious form of conjunctivitis ("pink-eye"). It is a Gram-negative small bacillus, usually intracellular, identical morphologically with the influenza bacillus.

The gonococcus is a common cause of acute severe conjunctivitis, both in the newborn and in adults with ordinary infection, but is rarely seen in young girls with vulvovaginitis. The organisms can be recognized as a rule in Gram-stained films, from their morphology and intracellular location. The secretion is usually abundant and there are usually no contaminating organisms. Gonococci must be differentiated from *Neisseria catarrhalis*, which may be present in the conjunctiva but rarely causes much inflammatory reaction. Gonorrhea, long after the acute urethritis, may cause an iritis in men (rarely in women).

The meningococcus may cause a severe conjunctivitis or panophthalmitis, usually in the course of a cerebrospinal meningitis.

The Morax-Axenfeld bacillus may cause a conjunctivitis which is commonly chronic, "dry," and most marked about the inner canthus. The organisms occur in pairs or short chains, usually extracellular or occasionally within phagocytes. They resemble the Friedländer bacillus but do not have capsules.

The Friedlander bacillus may cause a conjunctivitis or dacrocystitis, and is occasionally found in atrophic rhinitis when the nasal ducts are involved.

The pyocyaneus bacillus may cause a severe purulent keratitis as well as a conjunctivitis. The pyocyaneus toxin seems to be a factor in the production of the lesions.

In leprosy there may be conjunctival and corneal ulcerations. The bacilli are usually abundant in the discharge. In tuberculous lesions of the conjunctiva or lacrimal sac, organisms are usually sparse and animal inoculations or cultures may be necessary to demonstrate them.

Pasteurella tularensis may cause an ulcerative conjunctivitis with lymphadenitis of the cervical glands, fever, and other manifestations of systemic infection.

Iritis and iridocyclitis are most often attributed to a focal infection, but a syphilitic origin must be considered.

Choroidoretinitis, uveitis, and other inflammatory lesions are frequently associated with a strongly positive tuberculin reaction and are attributed by some to an allergic reaction to products liberated from tuberculous lesions in other organs.

In keratomycosis the cause has been ascribed to *Aspergillus fumigatus*.

Trachoma is now known to be due to a filtrable virus related to psittacosis. The trachoma bodies are a type of intracellular inclusion body.

Epidemic keratoconjunctivitis, a moderately contagious disease characterized by the development of punctate opaque spots in the cornea, is due to a filtrable virus.

A conjunctival irritation may be allergic in origin, and in such cases the smear from the secretion often shows the presence of eosinophils.

Animal Parasites. The larval form of *Taenia solium* (*Cysticercus cellulosae*) has a predilection for eye as well as brain. It is usually situated beneath the retina.

Echinococcus cysts have been reported in the orbit.

The adult *Loa loa* tends at times to appear under the conjunctiva or in the subcutaneous tissue of the eyelids.

The larvae of *Onchocerca volvulus* may be present (in chronic cases) in the cornea and other tissues of the eye, causing a characteristic punctate keratitis, iritis, and eventually blindness.

Fly larvae have been reported from the conjunctival sacs in the helpless sick, species of larval sarcophagids having been reported as invading the conjunctival region in purulent ophthalmias.

Demodex may cause an obstinate blepharitis.

CHAPTER 31

Diagnosis of Infections of the Mouth and Pharynx and of the Nose and Ear

Mouth and Pharynx

Normally the mouth and pharynx contain enormous numbers of bacteria of many kinds and frequently various protozoa and fungi. Many of these organisms are harmless saprophytes, some are potential pathogens, and sometimes virulent bacteria are harbored which the individual can resist sufficiently to prevent the development of clinical infection, but which may cause disease in others.

Bacteriologic studies of infections in this region are, therefore, difficult, and their interpretation requires considerable experience. Doubtless some of the pathogenic organisms present are in a dissociated phase, and their significance will be better understood when more is known about the variability of different species.

In certain conditions, however, bacteriologic studies are of value.

Staphylococci (aureus and albus), streptococci of different types, diphtheroid bacilli, pneumococci, sarcinae, colon and other Gram-negative bacilli are commonly found. The Friedländer bacillus, proteus, *Neisseria catarrhalis*, leptothrix, and various yeasts and molds occur. Spirilla and spirochetes are almost constantly present. In addition various organisms occur which have not yet been carefully studied and classified.

Material is obtained with a sterile, cotton-tipped wire or wood applicator. Sterile forceps may be convenient for obtaining particles of membrane. For making smears from around the gingival margins a sterile toothpick is useful. Antiseptic gargles or mouth washes should not be used for several hours before taking a culture.

Diphtheria. In individuals with clinical diphtheria the diphtheria bacilli are usually so abundant and unmistakable morphologically that their demonstration by culture and smear is practically diagnostic. Cultures should be made on Loeffler's medium, and a direct smear from the lesion should be stained and examined at once (see p. 93). Diphtheroid bacilli, which are almost always present in the normal throat, can usually be differentiated morphologically. Virulence tests may be necessary for a definite diagnosis.

Streptococcus Sore Throat. Smears from the inflamed area usually show large numbers of streptococci. Cultures should be made by streaking a blood agar plate on which hemolytic streptococci are easily distinguished from other types. If hemolytic streptococci are present in large numbers the culture has diagnostic value. Hemolytic streptococci are also constantly present in the angina of scarlet fever. However, they are present in smaller numbers in about 10 per cent of normal individuals, and the interpretation of their presence in small numbers is difficult.

Vincent's Infections. Ulcerative lesions of the mucous membranes associated with the fusiform bacilli and spirochetes described by Vincent contain enormous numbers of these organisms, which can be demonstrated readily in smears (see p. 157). In making these smears it is important to obtain material from the depths or margins of the ulcer. Being anaerobic organisms, they are more numerous under the membrane. If only a few are found, one should be skeptical about their etiologic significance, for they are commonly present in the normal mouth, especially around the teeth. Although it is possible to obtain cultures by special methods, this procedure is not feasible for diagnosis.

The bacteriologic diagnosis of atypical streptococcus and Vincent's infections is not always simple. Diphtheria ulcerations may be secondarily infected with these organisms. Since the treatment of these conditions is entirely different, all of these organisms should be searched for, and considerable judgment and clinical knowledge is necessary in interpreting the bacteriologic findings. In addition, streptococci, or fusiform bacilli and spirochetes, may be found in lesions of the mucous membrane that are due to other causes, such as carcinoma, leukemia, and agranulocytic angina. A blood examination may be necessary to exclude these conditions.

Syphilitic Ulcers. In syphilitic ulcers the *Treponema pallidum* may be demonstrated by darkfield illumination, but the presence of morphologically similar spirochetes in the normal mouth makes this procedure useless for diagnosis. The *Treponema pallidum* does not stain by the ordinary methylene blue or carbolfuchsin stains which serve to demonstrate the spirochetes of Vincent.

Tonsillitis. Tonsillitis may be caused by different species of bacteria. Staphylococci, streptococci, and occasionally influenza bacilli may cause severe inflammation. Aside from acute infections the tonsils may chronically harbor organisms in the depths of the crypts and in their substance, in which case they may act as a focus of infection. Tubercle bacilli have been found in sections of diseased tonsils.

Epidemic Meningitis. Meningococci are found in the nasopharynx in patients with epidemic meningitis, and in many contacts. In addition an appreciable number of normal individuals have been shown to be chronic carriers. For the demonstration, cultures should be taken from behind and above the soft palate with a bent wire swab, and the material should be immediately inoculated on blood or serum agar and quickly put in the thermostat.

Fungus Infections. The thrush fungus, *Candida albicans* (*Monilia albicans*), causes the development of firm, hard, creamy white patches on the mucous membrane. The fungus may be easily demonstrated by mounting a bit of the membrane in 10 per cent sodium hydrate, or by crushing and staining by Gram's method. The filaments are segmented and branching, and round or oval spores are seen between the filaments and attached to them in short budding chains. Other monilia may be found in sprue ulcerations about the tongue or buccal mucosa, and also in the feces.

Actinomycosis may develop about a carious tooth, and the finding of the ray fungus in the yellow granules from the pus establishes the diagnosis.

Animal Parasites. Amebae and flagellates have been reported from the mouth. *Endamoeba gingivalis* (*E. buccalis*) is frequently obtained from scrapings about affected teeth or in the contents of root abscesses. At present it is regarded as of no importance. In the remarkable disease "halzoun," flukes have been found to be the cause of the asphyxia.

Roundworms (*Ascaris*) may be vomited up and, lodging in the pharynx, may have to be extracted.

In the Mediterranean basin the leech, *Limnatis nilotica*, may gain access to the upper pharynx, through drinking water from springs and pools, and attach itself to the mucous membrane of the nasal and buccal cavities.

Nose

Material from the nasal cavities may be obtained for bacteriologic examination by washing about the alae with sterile water, then having the patient blow his nose on a piece of sterile gauze and taking the material for culture or smear from this. If the material is purulent and located at some ulcerating spot, or if a sinusitis is suspected, it is best to use a speculum, and either touch the spot or the orifice of the sinus with a sterile swab or use a capillary-bulb pipet with a slight bend at the end.

In general, the bacteria found in the nose are like those in the throat. Normally one finds chiefly white staphylococcus colonies and colonies of short-chain streptococci, *Neisseria catarrhalis*, occasionally *G. tetragena*, the xerosis bacillus, and Hoffmann's bacillus, *Corynebacterium pseudodiphtheriticum*.

The meningococcus, *H. influenzae* (Pfeiffer), and the pneumococcus have also been frequently found in cultures from the nasal secretions.

In some cases of atrophic rhinitis an organism of the Friedländer type may be obtained in pure culture.

Diphtheria involving the nasal cavity must always be kept in mind, and in quarantine investigations the examinations of the nasal secretions culturally should be a part of the routine.

The tubercle bacillus may be found in nasal ulcerations; it is, however, present only in exceedingly small numbers. On the other hand, one of the best diagnostic procedures in leprosy is to examine repeatedly smears from nasal mucous membranes for the *M. leprae*. In such ulcerations the bacilli are found in the greatest profusion. Rarely glanders may cause ulcerations.

Proteus vulgaris is frequently responsible for the production of foul odors in nasal discharges but does not seem to produce inflammatory conditions of the nasal mucosa. It simply decomposes the discharges. Various fungi have been reported from the nose, but in such a region the strictest conservatism in interpretation should be observed. The spores and mature sporangia of the fungus Rhinosporidium may be found in the nasal mucus in cases of this infection.

So many degenerative changes in epithelial cells resemble protozoal forms that such findings require ample confirmation.

The larval form of *Linguatula serrata* is a rare parasite of the nasal cavities; it is not infrequent, however, in the nostrils of dogs.

Various fly larvae are far more common, and the "screw worm," the larva of the *Cochliomyia americana*, is common in certain parts of tropical America, and may cause death by the effects of its burrowing.

The larvae of *Sarcophaga* have in particular been found in the nasal cavities of children. Myriapods, while of very little importance elsewhere, have been reported more than 30 times from the nasal fossae.

Ear

The middle ear is normally free of bacteria, but in affections of the throat, as with streptococci, pneumococci, and diphtheria bacilli, these organisms may infect it by way of the Eustachian tube.

Otitis Media and Mastoiditis. In a study of the bacteriology of otitis media, in 277 cases, Libman and Celler found streptococci present alone in 81 per cent, *Streptococcus mucosus* in 10 per cent and pneumococci in 8 per cent; staphylococci and Pyocyaneus and Proteus bacilli have also been found. Mixed infections are common. A streptococcus of the hemolytic group is a frequent cause of mastoid infections and of sinus thrombosis and brain abscess.

The influenza bacillus of Pfeiffer, nonvirulent diphtheroid bacilli, the meningococcus, the typhoid bacillus, *E. coli, N. catarrhalis, G. tetragena,* and the Friedländer bacillus have been cultivated from discharges from the middle ear and mastoid.

Affections of External Auditory Canal. The molds are of greater importance in affections of the external auditory canal than the bacteria. The cerumen seems to make a good culture medium so that various species of Aspergillus, Mucor, etc., develop and may obstruct the canal. These infections are often introduced by the patient's finger. Various mites and fly larvae have been reported from the ear.

Aural Myiases. The "screw worm," the larva of *Cochliomyia americana,* is the most common cause of aural myiasis in tropical America. The fly deposits its eggs about aural and nasal cavities of those with offensive discharges. The larvae gain entrance to the cavities of the head and develop, causing intense pain and giddiness. Larvae of *Sarcophaga, Calliphora,* and *Anthomyia* have also been reported from the external auditory meatus. The tympanic membrane may be perforated by them.

CHAPTER 32

Examination of Sputum

In the collection of sputum for examination great care must be taken that the material is actually coughed up from the bronchi or lungs. Secretions from the mouth or nasopharynx are worthless for examination. Sputum should be obtained if possible in the early morning, just after awakening, and the patient should be instructed to save only what is raised from the chest. It is desirable to have the patient clean the mouth thoroughly before obtaining the specimen. Material from the mouth and pharynx can usually be recognized by its appearance, and by the presence of mosaic-like groups of flat epithelial cells. Material from the bronchi or lungs is either frothy mucus or mucopurulent material, and may contain alveolar cells as well as pus cells.

Macroscopic Examination. The gross appearance of the sputum is of importance. Large amounts may be expectorated constantly in bronchiectasis, or in tuberculosis with cavity formation. A large amount appearing suddenly suggests rupture of an abscess of the lung, pleura, or liver. A sudden profuse expectoration of frothy, serous material, which may be pinkish, salmon-colored, or frankly blood-tinged, suggests an acute pulmonary edema that demands immediate treatment.

The color may be white, yellowish, or greenish, depending in part upon the amount of pus present. Bright-green sputa occur in resolving pneumonia and in jaundice. The rusty sputum of pneumonia is familiar to everyone. Bright blood suggests tuberculosis, but may occur in carcinoma, bronchiectasis, abscess, and other conditions. Bleeding in the mouth or nasopharynx may simulate hemoptysis.

Ordinarily the sputum is odorless, but in bronchiectasis, cavities, abscess, and gangrene, and in fusospirochetosis the odor may be very foul.

Dittrich's plugs are caseous yellowish or gray plugs formed in the bronchi in bronchiectasis or putrid bronchitis. They have a very foul odor when crushed. Occasionally fibrinous casts of the smaller bronchi may be coughed up in pneumonia or in fibrinous bronchitis.

Curschmann's spirals may be seen as whitish, curled threads, which, under the low-power objective of the microscope, appear as central threads surrounded by coils of fine fibrils. They occur chiefly in bronchial asthma.

Microscopic Examination. The examination of an unstained preparation is of great value although often neglected. Small purulent or cheesy particles are selected and flattened out in a thin layer under a coverglass. These are examined for elastic tissue, heart-failure cells, fungi, amebae, and ova of animal parasites. The addition of 10 per cent sodium hydrate to the preparation facilitates the examination for elastic tissue and fungi.

Elastic fibers are highly refractile, wavy fibrils of uniform diameter, often split or frayed at the ends. Unless they show an alveolar arrangement one cannot be certain that they have not come from the food. They occur in destructive diseases of the lungs such as advanced tuberculosis, abscess, and gangrene.

Heart-failure cells are large mononuclear cells, possibly endothelial leukocytes, filled with brownish or yellowish granules of blood pigment. The nucleus is usually eccentric, but may be obscured by the pigment granules. They occur in chronic passive congestion of the lung, associated most frequently with mitral stenosis, and also following any type of hemorrhage into the lung. Similar cells filled with blackish granules occur in individuals exposed to a dusty atmosphere. Myelin globules also occur in such cells, but they have no special significance.

Charcot-Leyden crystals are frequently present in bronchial asthma, often adhering to the Curschmann's spirals. They are colorless, thin, pointed, hexagonal crystals. They are present in conditions in which eosinophils occur, and there seems to be some association between them. They are often found in paragonimiasis, and in the pus of amebic abscesses discharging through the lungs. Other crystals may occur—fatty-acid crystals (especially in Dittrich's plugs), hematoidin, cholesterol.

Fungi. *Actinomyces* (ray fungus) is found in actinomycosis of the lung. The yellow "sulfur" granules can usually be seen with the naked eye, and under the low-power objective of the microscope appear as finely granular bodies. After crushing under a coverglass and examining with the high-power lens, the masses of mycelial threads arranged radially around the edges and ending in characteristic bulbous tips can be seen. *Aspergillus fumigatus* may rarely cause a pulmonary mycosis resembling tuberculosis, but is also found as a contaminant in old sputum specimens. These organisms may be recognized by their sterigmata carrying chains of spores. *Streptothrix* infections of the lung occur. The organisms have long, branching filamentous forms with small swollen buds at the ends resembling spores. *Blastomyces* causes a fatal infection. Blastomycetes are round or oval, doubly contoured, refractile, yeastlike cells which show budding. In cultures hyphae are formed, but in the tissues only budding forms are present. *Coccidioides immitis* may be found in the sputum of patients with pulmonary lesions as a spherical body about 30μ in diameter, with a highly refractile cell wall enclosing many small endospores. It never shows budding or hypha formation in the sputum. *Monilia* occur as contaminants, but may also cause pulmonary disease. Both yeast forms and mycelial filaments are found in the sputum. Spores are formed at the tips of the mycelia and at the mycelial nodes.

These fungi are discussed in detail in the section on fungi. Their presence in the sputum should be confirmed by repeated examination of fresh specimens before etiologic significance is attached to them, since many fungi occur as contaminants. They can also be seen in stained films, but their structure is better brought out in the fresh preparations. Cultures from the sputum can be made on Sabouraud's medium for further identification, but it is often difficult to obtain pure cultures.

Stained Smears. For the study of the cells of the sputum one can use one of the Romanowsky stains, or hematoxylin and eosin. The eosinophils which are so characteristic of bronchial asthma are well brought out. In sputum from cancer of the lungs clusters of unusual cells may be found.

Examination for Tubercle Bacilli. To make smears for staining, the sputum should be poured into a Petri dish which is placed over a dark background. Several suspicious particles are picked out with forceps or toothpicks and smeared on slides. Small, opaque, grayish or yellowish cheesy masses, or purulent streaks are selected. These are dried and fixed with heat and then also with absolute alcohol. As a routine measure, sputum should be stained by the Ziehl-Neelsen method and by Gram's method.

The finding of acid-fast bacilli is good evidence of the presence of tuberculosis, since nonpathogenic acid-fast organisms do not ordinarily occur in the sputum.

CONCENTRATION METHODS. When the number of tubercle bacilli in the sputum is relatively small they may be demonstrated more easily by one of the various concentration methods (see p. 82). If sputum is abundant and bacilli are not found, the total quantity should be collected for 24 to 72 hours and examined after concentration. If a suitable specimen of sputum is not obtained, the fasting stomach washings should be examined in a similar manner.

Without the use of concentration procedures, cultures, or guinea-pig inoculation, negative findings are entirely inconclusive.

SPIROCHETES AND FUSIFORM BACILLI. These organisms may be demonstrated in stained films, or by darkfield illumination. Before obtaining the sputum it is desirable that the mouth and teeth be thoroughly cleansed to avoid as much as possible contamination from this source. The sputum must be examined when perfectly fresh since the spirochetes disintegrate rapidly on standing. Special spirochete stains are not necessary, since the organisms stain well with carbolfuchsin 1:5, heated gently over a flame for a few minutes, or with Loeffler's methylene blue for 5 to 10 minutes. The characteristic fusiform bacilli stain easily with any method. In fusospirochetal infections they are constantly present in large numbers. If only a few organisms are found, one should be cautious in their interpretation, since they are frequently present in the mouth. Other organisms, especially (aerobic and anaerobic) streptococci, are commonly found with them, and their pathogenicity alone is questioned.

PNEUMOCOCCI. Pneumococci are best demonstrated by a Gram stain. In sputum from patients with lobar pneumonia they are often present in large numbers, and when surrounded by a definite capsule their presence may be considered significant. Methods for typing them from the sputum are given in the section on the pneumococcus. However, pneumococci occur often in sputum from conditions other than pneumonia, and are frequently present in the normal mouth.

OTHER ORGANISMS. For the demonstration of other common organisms in stained preparations it is desirable to wash the sputum if possible before making the films, as in making sputum cultures, in order to lessen the number of contaminating bacteria from the mouth. Even in such a preparation the presence of some organism is significant only if it occurs in great preponderance. Such organisms as the streptococcus, pneumococcus, influenza bacillus, staphylococcus, Friedländer bacillus, and *N. catarrhalis* may be recognized in a Gram stain, or preferably by culture. They are found in a variety of inflammatory conditions in the bronchi and lungs, sometimes as the etiologic agent, and at other times as secondary invaders. These organisms, together with various saprophytic bacteria, are often present in tuberculous lesions, especially cavities. Their significance, therefore, can only be interpreted by considering the clinical aspects of the case.

Sputum Cultures. A tenacious, mucopurulent mass is selected, and placed in a dish of sterile salt solution in which it is gently agitated. This process is repeated in as many changes of salt solution as its consistency permits. The particle is then placed at one side of a blood agar plate, and thoroughly broken up with a platinum loop. The surface of the plate is then streaked over with the loop or a bent platinum wire. If the sputum could not be thoroughly washed or if organisms are numerous, a second plate should be streaked without recharging the wire in order to obtain discrete colonies. In this way a practically pure culture may sometimes be obtained.

Cultures of *H. pertussis* are obtained more readily by holding an open plate of the Bordet-Gengou medium about 4 inches in front of the mouth of a patient with whooping cough during a paroxysm of coughing. The organism may be obtained in this way in a large percentage of cases in the early stages.

For culturing the *tubercle bacillus* a special technic is necessary. After digestion by Hank's method (see p. 82) or some similar procedure, and neutralization, the sediment is inoculated on an egg-yolk medium or some other special medium (see p. 347).

The blood-stained, watery sputum of *plague pneumonia* should be cultured on plates of plain agar and on 3 per cent salt agar at the same time. An ordinary smear stained with carbol thionin, however, practically enables one to establish a diagnosis. A guinea pig should be inoculated cutaneously.

Fungi should be cultured on Sabouraud media as described in the section on fungi.

Animal Inoculation. The inoculation of a mouse at the root of the tail or intraperitoneally is a useful method for isolating pneumococci from the sputum. Pure cultures can usually be obtained from the heart's blood, and an emulsion of the peritoneal exudate can be used for typing. The details of this procedure are given in the section on the pneumococcus.

For the demonstration of tubercle bacilli, contaminating bacteria should be killed by one of the digestion methods. The pig is injected subcutaneously in the groin and watched for from three to six weeks. The characteristic lesions are described in the section on the tubercle bacillus.

Guinea pigs may be infected with plague bacilli by simply rubbing the infected material on the shaven skin.

Rivers (1935) reports the production of characteristic intracellular bodies in the spleens of mice injected with psittacosis sputum either filtered, or unfiltered if no pneumococci or streptococci are present. Intranasal inoculation of mice, hamsters, or cotton rats may be used for isolation of many of the respiratory viruses.

Vaccines. The value of vaccine therapy in the acute respiratory infections is dubious, but in chronic infections, especially in chronic bronchitis associated with asthma, autogenous vaccines sometimes seem to be of benefit.

The organisms most frequently obtained from the sputum in these cases are the streptococcus (viridans or hemolyticus), the pneumococcus (rarely one of the fixed types), and the influenza bacillus. Other varieties mentioned above are less commonly present. In preparing a vaccine, smears and cultures must be examined with great care to determine the predominant type of organisms. If more than one pathogenic species is present in the culture in large numbers a mixed vaccine can be used. Intracutaneous tests for hypersensitiveness to the vaccine are sometimes used to determine the pathologic significance of the organism.

Animal Parasites. Amebae from a liver abscess rupturing into the lung may be found. Pulmonary infection with *Paragonimus ringeri* is very important. This is recognized by the presence of operculated eggs in the sputum.

Hydatid cysts, either of the lung, or of the liver rupturing into the lung, may be recognized by the presence of *Echinococcus* hooklets. The material is bile-stained if from the liver. Dutcher has reported filarial embryos from sputum.

The larval forms of hookworms, *Strongyloides* and *Ascaris* have been found in sputum at time of migration, but examination for these is not practicable as a diagnostic procedure.

CHAPTER 33

Examination of Pus

Pus may be collected for examination either (1) with a platinum loop, (2) with a sterile swab, (3) with a bacteriologic pipet or (4) with a hypodermic syringe.

It is always well to make a smear and stain it by Gram's method at the same time that cultures are made. The Gram stain gives information as to the abundance of organisms in the pus and as to the probable findings in the culture. Pneumococci and streptococci are differentiated from the staphylococci in this way without the necessity of extended cultural methods.

The hypodermic syringe is very useful in puncturing buboes, etc., especially in plague. A small pledget of cotton on a toothpick dipped into pure carbolic acid and touched to a spot over the bubo, the escharotic action being arrested with alcohol after about 30 seconds, makes a sterile anesthetic spot at which to introduce the needle of the syringe.

A bacteriologic pipet is very useful when pus is to be sent to a laboratory; the tip can be sealed in a flame and the cotton plug at the other end insures the noncontamination of the contents. The material may be drawn up either with the mouth or with a rubber bulb.

Smears from material examined for gonococci may show Gram-negative diplococci which, however, may not have the typical morphology of the *Gonococcus*. They are furthermore often extracellular. *N. catarrhalis* has been reported from urethral smears though very rarely. Diphtheroid organisms are not uncommon. Gram-positive cocci are rather common in smears from discharges of chronic gonorrhea.

In a study of the aerobic bacterial flora of war wounds Lawrence found that more than 80 per cent of the discharges from such wounds showed streptococci, which especially flourished in deep pockets, staphylococci replacing them in shallow wounds. Gram-negative bacilli were present in 95 per cent of smears. Of these, *E. coli* was present in 50 per cent of cases. The combination of aerobes and anaerobes in a wound makes conditions more favorable for the anaerobes. Wounds contaminated with fusiform bacilli do badly.

The pus from wounds infected with anaerobes is usually very foul. The most important anaerobe in the discharge from gas gangrene wounds is *Cl. perfringens*.

The pus from the necrotic center of climatic bubo is sterile.

It is remarkable how frequently pure cultures are obtained from abscess material. In purulent material from abdominal abscess we are apt to obtain mixed cultures, especially the colon bacillus and pyocyaneus bacilli, in addition to ordinary pus organisms.

In examining blood-serum or blood-agar slants inoculated with purulent material, the water of condensation is always examined for streptococci.

When the plague buboes begin to soften, the plague bacilli may be replaced by ordinary pus organisms.

Animal inoculation also is often necessary in plague and glanders, and sometimes in anthrax. When tetanus is suspected, it should be searched for as described under Tetanus. Tuberculosis should be identified by inoculating a guinea pig, as well as by acid-fast staining and culture, if there is any doubt as to the nature of the material.

The black or yellow granules of Madura foot, as well as those of actinomycosis, should be examined as recommended in the section on fungi.

Amebae, coccidia, and larval echinococci may be found in purulent material, as may also various other animal parasites, as fly larvae, sarcopsyllae, etc.

The pus from an amebic abscess of the liver is as a rule sterile when cultured, and the examination at the time of operation or exploration frequently shows an absence of amebae as well as of bacteria; but two or three days later amebae may be found in the pus draining from the abscess cavity.

Flukes, roundworms and whipworms may cause abscesses as a result of their wandering from the intestinal lumen.

Serious ulcerations may follow infection with the guinea worm.

Abscesses often occur about encysted filarial worms.

CHAPTER 34

Skin Infections

Cultures should be made, as a rule, in the bacteriologic examination of lesions of the skin. The surface should first be washed with soap and water in order to eliminate chance organisms from dust or other extraneous sources. Scrapings are then made with a sterile dull scalpel, and the material obtained is emulsified in a drop of sterile water in a Petri dish. A tube of melted agar at 42° C. may be poured over the drop and mixed, or the material may be spread over the surface of an agar plate. About 80 per cent of the colonies developing will be staphylococci and the greater proportion will be white colonies.

Occasionally *S. aureus* or *S. citreus* may be isolated. *S. aureus* is the organism usually isolated from furuncles, circumscribed abscesses, and carbuncles. Streptococci and colon bacilli are rarely found. Streptococci are the organisms to be expected in phlegmonous infections. Cold abscesses, which are frequently due to tuberculous infection, as a rule are sterile. Acne pustules may show staphylococci, the acne bacillus, or both.

Corynebacterium acnes (*Bacillus acnes*) is a short, usually pleomorphic bacilius about 1.5 × 0.5μ in size, which often shows a beaded appearance when stained by Gram's method. It is Gram-positive. It grows readily on glucose agar when cultivated anaerobically, and also aerobically on acid media containing blood serum. Colonies appear in four to five days.

The "bottle bacillus" (*Malassezia ovalis*), which has the morphology of a yeast, is regarded as the cause of dry pityriasis capitis. It may also be found in the comedones of children.

In the tropics an organism which is probably a virulent strain of *Staphylococcus aureus* at times produces lesions similar to impetigo, at other times pemphigoid eruptions or wide-spreading erysipelatous conditions. It has been described under the name of *Diplococcus pemphigi contagiosi*.

Staphylococcus epidermidis, the common cause of stitch abscesses, is considered by Sabouraud to be the cause of eczema seborrhoeicum.

In scrapings from the skin of lepromata the acid-fast bacilli are found in the greatest profusion. In tuberculosis of the skin the tubercle bacilli are exceedingly scarce. Inoculation of a guinea pig usually gives positive results with the tubercle bacillus. The leprosy bacillus is nonpathogenic for laboratory animals.

Anthrax and glanders cause skin lesions which can be surely diagnosed only culturally or by animal inoculation.

Plague bacilli may be isolated from the primary vesicles appearing at the site of the flea bite.

Tropical phagedena is thought by some to be due to a sort of diphtheroid organism. The organisms of Vincent's angina may cause tropical ulcer.

In granuloma venereum in scrapings from the ulcerations on the external genitals and

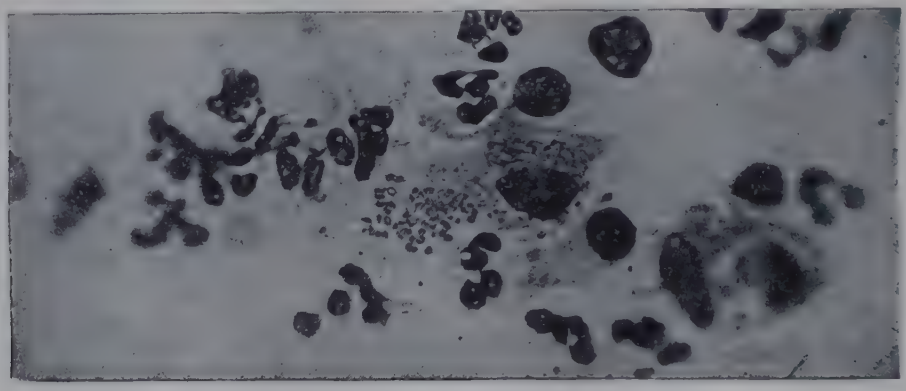
adjacent skin, macrophages containing numerous small bacilli, *Donovania granulomatis,* may be found (see p. 148).

The skin diseases due to fungi, which are widely prevalent, are discussed in Chapter 9.

Animal Parasites. Certain skin diseases, as oriental sore and the cutaneous lesions of uta or espundia, are caused by a *Leishmania.*

Of the skin eruptions caused by animal parasites, ground itch (dew itch, foot itch) is the most important. This is a dermatitis caused by the irritation set up by the hookworm larvae penetrating the skin of the foot and leg.

According to Lemaire, 10 species of nematode larvae, other than those parasitic in man, may penetrate the skin, setting up a dermatitis. Not being adapted to man they



Donovania granulomatis. In a scraping from an ulcer from a case of granuloma venereum. Tropical Institute, Leiden. (After Flu, from Ruge, Muhlans, and zur Verth.)

die out without infecting other tissues. One type of such dermatitis is the "creeping eruption" caused by larvae of the dog hookworm. A similar eruption is caused by larvae of the bot fly of horses (*Gastrophilus*).

A dermatitis is produced by the penetration of cercariae of *Schistosoma* into the skin.

Filarial infections are also important, especially the ulcers of the guinea worm, Calabar swellings of *Loa loa,* the cystic tumors of *Onchocerca volvulus* and the varicose groin glands and elephantiasis of *W. bancrofti.*

Enterobius (*Oxyuris*) may cause a severe irritation about the region of the groin and inner surfaces of the thigh, and especially about the vulvar region of female children.

The larvae of *Gnathostoma spinigerum,* a nematode with two liplike structures and spinelike appendages covering its anterior one-third, have been found in tumefactions of the skin.

Plerocercoid larvae of Diphyllobothriidae have been found in the subcutaneous tissues.

Leeches, as *H. zeylanica,* may cause serious ulceration.

The *Tunga penetrans* (*Sarcopsylla penetrans*) or jigger (sand flea) is an important agent in ulcerations about the foot.

Certain acarines cause skin lesions, as do also the larvae of certain flies (see Chapter 27).

The itch mite (*Sarcoptes scabiei*) is an important animal parasite of the skin.

The various lice, fleas, and bedbugs are well-recognized causes of skin irritation.

CHAPTER 35

Examination of the Cerebrospinal Fluid and of Fluid from the Serous Cavities

Cerebrospinal Fluid

The average normal volume of cerebrospinal fluid is 100 to 150 ml. The normal cerebrospinal fluid pressure with the patient on his side in the recumbent position is 5 to 12 mm. of mercury or 70 to 160 mm. of water. Cerebrospinal fluid pressure above 15 mm. of mercury or 200 mm. of water is considered increased. The increase in cerebrospinal fluid pressure may be due to any one of four factors: (1) increased rate of secretion, (2) decreased rate of absorption, (3) loss of continuity of the subarachnoid space or obstruction of the connecting foramina, or (4) increase in brain volume. The rate of secretion is increased in all inflammatory conditions, and unless the rate of absorption keeps pace with the rate of secretion there results an increased volume of cerebrospinal fluid and an increase in pressure. In acute inflammatory conditions the rate of absorption lags behind the rate of secretion, thus producing an increased pressure. If there is any block or obstruction of the subarachnoid space, the cerebrospinal fluid pressure rises cephalad to the block. Increase in cerebrospinal fluid pressure may occur with brain tumors or abscesses in certain locations in the brain.

Lumbar Puncture

An appropriate intervertebral space, either the third or fourth lumbar, is selected. The overlying skin is anesthetized, an especially sharp, small needle being used for the purpose. A nonbreakable, sterilized, sharp, short-beveled spinal-puncture needle with an accurately fitting stylet is selected. The spot in the anesthetized area chosen for puncture is marked with the thumbnail of the operator's gloved left hand, then the needle is passed gently and slowly but firmly in the midline and straight in. After the skin has been pierced, the needle is realigned and the fixed supraspinous ligament is entered. This is probably the most important step in a successful puncture, as the direction of the needle is very difficult to alter after entering this structure. The needle will pass with ease and practically no resistance the loose areolar tissue which is next encountered. The second point of resistance will be the ligamentum flavum. This ligament produces sense of resistance and the stylet may be withdrawn at this time. If no fluid is obtained the stylet is reinserted and again the needle is pushed a millimeter or two further where one again meets resistance on puncturing the dura. As the needle punctures this inelastic membrane a definite sense of "give way" and palpable "click" is felt. The stylet is removed and if the lumen of the needle is in the subarachnoid space, cerebrospinal fluid will be obtained. If no fluid is obtained the needle is rotated a half turn, the stylet is again removed, and observation made for flow of fluid.

This procedure of withdrawing stylet may be performed from time to time during passage of the needle if one is not certain that the needle point is in the canal. The manometer should be in readiness and before the stylet is withdrawn completely the

spinal-fluid pressure reading should be taken. It is best to rotate the needle and to take the pressure reading a second time as the lumen may be partly blocked or only partly in the canal. This is especially important in all acutely ill patients and those suspected of increased intracranial pressure. In the case of "dry tap" the needle may be rotated, slightly withdrawn, or pushed in a little as no flow usually means that the needle point is not in the subarachnoid space.

If one inserts the needle too far the venous plexus on the anterior wall may be cut and bloody fluid obtained. If the needle is withdrawn a very short distance this bleeding will usually cease after a few milliliters of fluid have been obtained. The second portion of fluid, if clear, may be sent to the laboratory for examination.

A "bloody tap" is usually due to trauma to a vessel. As a rule the first portion of fluid removed contains more blood than the last. A bloody fluid is practically useless except for a Wassermann reaction and for cultures. If the fluid is only slightly blood-tinged, however, it may be possible to demonstrate an increase in the cell count by making total counts of both red and white cells, and subtracting from the latter the number of leukocytes estimated to have been introduced with the blood (1 for each 750 red cells). A second diagnostic puncture as a rule should be postponed for 10 days, until the meningeal reaction to the puncture has subsided.

A fluid uniformly bloody in all fractions usually indicates a subarachnoid hemorrhage, or an intracerebral hemorrhage which has ruptured into the ventricles.

The patient should be kept prone in bed for 36 hours after the puncture to lessen the tendency to leakage of cerebrospinal fluid into the tissues through the puncture wound in the meninges. When the fluid does leak into the tissues an incapacitating headache will occur as soon as the patient sits up.

Caution. *Removal of spinal fluid is dangerous in patients with increased intracranial pressure due to brain tumor.* An ophthalmoscopic examination should be made as a routine procedure before every lumbar puncture. Any evidence of swelling of the optic discs calls for an exhaustive neurologic examination, and if a puncture is done, the fluid should be removed very slowly and the procedure stopped at the first indication of respiratory disturbance. Lowering a pathologically high pressure is beneficial (at least temporarily) in meningitis and in most other conditions.

The intravenous injection of hypertonic glucose or saline solution will usually produce a marked lowering of the cerebrospinal fluid pressure. For this purpose the use of 100 to 200 ml. of 50 per cent glucose solution intravenously has been found safe and efficient. The next best is 20 per cent sodium chloride solution. The vein above the point of injection of these solutions must not be compressed, as each in this strength is a sclerosing agent. Following the intravenous injection of such a hypertonic solution the cerebrospinal fluid pressure, after a sharp but very brief rise, falls profoundly for a period of three to five hours, and the former pressure is not again reached until about seven to eight hours later. The venous pressure at first also shows a sharp, sudden temporary rise, to return to its previous level. The arterial pressure is not notably disturbed.

Queckenstedt's Test. This test is valuable in suspected subarachnoid block and in lateral sinus thrombosis. With a needle in the subarachnoid space and a manometer attached, an assistant compresses both jugulars and the rapidity of the rise in pressure is observed. In a normal individual the cerebrospinal fluid pressure is rapidly increased. Failure to obtain a prompt and rapid rise in pressure on compression of both jugulars indicates a subarachnoid block. A similar marked rise in pressure obtained by compression of one jugular vein only indicates obstruction of the lateral sinus on the opposite side (usually thrombosis, rarely an anatomic anomaly).

Appearance of the Cerebrospinal Fluid

Normal cerebrospinal fluid is crystal clear and colorless. There is no pellicle, clot, or sediment of any kind. An increase in the cell content of the cerebrospinal fluid will

produce a loss of transparency varying from slight opalescence to gross turbidity depending upon the number and type of cells present. As a rule it takes a larger number of lymphocytes (500 or more) than polymorphonuclear leukocytes (200 or more) to produce turbidity. The cerebrospinal fluid is usually described as clear, opalescent, turbid, or purulent.

Coagulation. The normal cerebrospinal fluid contains no fibrinogen and therefore it does not coagulate. Any infectious condition which allows cells and fibrinogen to pass into the spinal fluid permits coagulation to take place when the fluid is exposed to the atmosphere. A fine, fragile, cobweb-like pellicle suggests tuberculous meningitis; a clump-like coagulum suggests a suppurative condition; a fine precipitate-like coagulum is often seen in syphilis.

Erythrochromemia. This term is used to describe a red or reddish fluid due to blood or hemoglobin. If the red color is due to fresh blood, when the fluid is centrifuged the supernatant fluid will be clear, and the red cells will be packed in the bottom of the tube. In old hemorrhage the supernatant fluid is yellow.

Xanthochromia. This term is used to designate a yellowish color due to altered hemoglobin in the fluid, with no red blood cells. The explanation usually given is that the spinal veins have been compressed, and exudation of protein, corpuscles, and plasma has taken place. The hemoglobin disintegrates giving rise to a yellow color, there is a marked increase in protein, and the fluid coagulates spontaneously (Froin's syndrome). Xanthochromia is not diagnostic of any specific lesion, since tumors of the cord, tuberculous meningitis, extensive adhesions, gumma, or fracture of the spine may obliterate the continuity of the subarachnoid space and produce xanthochromia below the lesion.

The fluid may have a green or gray color in suppurative cases; and in long-continued jaundice there may be sufficient bilirubin present to give a yellow color.

Cisterna Puncture

Position of the Patient. The patient should be recumbent in bed on his right side, with the head supported by a pillow of correct thickness to secure alignment of the cervical with the thoracic vertebrae. The head is flexed as much as possible without producing pain or discomfort.

Operative Procedure. Except for children no anesthetic is ordinarily needed. A small nonbreakable cisternal-puncture needle is used. The thumb of the left hand is placed on the superior occipital protuberance and is caused to slide gently but firmly down the back of the neck until it encounters the bony prominence of the spine of the axis. This space is then marked with iodine, and the associated anatomic structures which guide in reaching the cisterna are carefully explored and visually brought into relation with each other for use in securing correct alignment and direction of the puncture needle as follows:

1. The cervical vertebrae are placed in line with the external occipital protuberance and thoracic vertebrae.
2. The head is flexed as much as possible without producing pain or discomfort.
3. The external auditory canal is definitely marked with a tightly rolled pencil of cotton.
4. The chin, nose, and sternum are kept in line by an assistant.
5. The glabella, external auditory canal, and needle are now brought into one continuous line.

The needle is thrust through the skin in the midline of the neck over the axis and its full length kept parallel with the external auditory canal. Keeping the needle in midline, the operator proceeds cautiously thrusting the needle forward and upward with the point toward the glabella. Proceeding thus, with the landmarks kept correctly aligned, the needle passes through the anatomic structures without meeting bony resistance. After penetrating to a depth of about 3 cm. the operator must proceed further with due cau-

tion until, as Ayer so aptly describes, he "receives the same sense of 'give way' as experienced in going through the lumbar dura." If in doubt, the stylet should be removed from time to time and the needle observed for flow of fluid. The needle should never be inserted more than 6 cm. in adults and never more than 3.5 cm. in infants. The cisterna is usually reached in adults at a depth of from 4 to 5 cm., and in infants from 2 to 3.5 cm. and about 1 to 1.5 cm. at the lower level. It is needless to say that one should secure practice on the cadaver.

Diagnostic Indications for Cisterna Puncture Only. The following are listed:

1. Suspected subarachnoid block.
2. Localized spinal block by using combined cisternal and lumbar puncture.
3. To localize lesions by injection of air or opaque material for roentgenograms.
4. Treatment of patients with specific serum, who may be losing ground under injections by lumbar puncture.
5. To relieve headache in block.

Technic of Examination of the Cerebrospinal Fluid

Cell Count. The cell count should be made as soon as possible after the fluid has been obtained. If the fluid is cloudy a trace of oxalate is added to a portion to prevent clotting. The fluid is shaken up, and a drop may be mounted on a hemacytometer chamber. It is preferable to use a staining solution such as:

Crystal violet, 0.1 Gm.; glacial acetic acid, 1 ml.; water to 50 ml.; 1 drop of phenol.

The staining solution is drawn to the mark 1 in a leukocyte diluting pipet and the pipet filled with spinal fluid to the mark 11. The contents of the pipet are shaken, a drop is mounted on a counting chamber, and the cells are counted as in making a leukocyte count. To correct for the dilution the count is multiplied by $\frac{10}{9}$.

Differential Count. A portion of the fluid is centrifuged, the fluid is decanted, and thin films are made from the sediment. These may be stained by Wright's stain, or they may be fixed with absolute methyl alcohol and stained with aqueous methylene blue. It is necessary only to differentiate polymorphonuclear leukocytes, lymphocytes, and large mononuclear endothelial cells. This can be done fairly well in the counting chamber.

Cytology. In fresh fluid the average number of cells in normal individuals over five years of age is 1 to 6 per cu. mm. A slight increase may be allowed for children under five years of age. Counts ranging from 6 to 10 are regarded as suspicious, and counts above 10 are definitely pathologic. The only cell found in normal cerebrospinal fluid is the small lymphocyte.

An increase in cells in the cerebrospinal fluid is usually an indication of an inflammatory condition. An inflammation due to cocci is attended by an increase in polymorphonuclear leukocytes, whereas viruses, treponemas, and tubercle bacilli usually produce a lymphocytic response. Irritation by chemicals, inflammation of neighboring structures (mastoid, sinuses, etc.), and the introduction of foreign protein into the subarachnoid space will also produce a mononuclear cellular increase.

Globulin. An increase in globulin may be demonstrated by:

1. Ross-Jones Test (Nonne-Apelt Reaction, Phase 1). Over 1 ml. of saturated ammonium sulfate solution is carefully layered 0.5 ml. of clear spinal fluid. An increase in globulin is indicated by the appearance of a thin white "ring" within a few seconds. (A ring may appear after five minutes or more with a normal fluid.)

2. Pandy's Test. To 1 ml. of a clear saturated solution of phenol in water (about a 10 per cent solution) a drop of spinal fluid is added. An increase in globulin is indicated by the appearance of a bluish-white cloud. A normal fluid may show a faint turbidity. The density of the cloud measures roughly ($+^1$ to $+^4$) the degree of increase in globulin.

Either test will give a positive reaction with a normal spinal fluid which is demonstrably contaminated with blood.

Total Protein. A quantitative estimation of the total protein in the spinal fluid is important, both for diagnosis and as an aid in estimating the effect of treatment in cases of syphilis.

Method of Denis and Ayer. Into a test tube of about 4-ml. capacity, 0.6 ml. spinal fluid is measured. To this is added 0.4 ml. distilled water and 1 ml. of a 5 per cent solution of sulfosalicylic acid. The contents of the tube are then mixed by inversion (but not by violent shaking) and after being allowed to stand for five minutes the suspension is read by means of a suitable colorimeter against a standard protein suspension prepared at the same time as the unknown. Before reading the standard against the unknown the standard solution should be placed in both cups and several readings made. In fluids of extremely high protein content, such as may be encountered in cases of spinal-cord compression and of meningitis, it is sometimes necessary to make a preliminary dilution with water as even 0.1 ml. of such fluids may contain too much protein to read against the standard.

Preparation of Standard for Determination of Total Protein (Ayer and Foster). Twenty ml. of fresh normal human blood serum are diluted to 200 ml. with 15 per cent solution of sodium chloride in a volumetric flask and filtered. This filtrate is the concentrated standard.

The total nitrogen of this filtrate is determined by macro-Kjeldahl on 40 ml. The nonprotein nitrogen is determined on the original undiluted serum by the micro-Kjeldahl method of Folin and this figure divided by 10 is subtracted from the total nitrogen to obtain protein nitrogen. Protein nitrogen multiplied by 6.25 gives the protein content of the concentrated standard.

This concentrated standard is diluted with distilled water to make the dilute standard containing 30 mg. protein per 100 ml.

These standards are preserved with a few crystals of thymol and kept on ice except when in use. In this way we have kept the concentrated standards for more than six and the dilute standards more than 12 months without appreciable change in protein content.

The standards prepared for estimation of albumin in the urine may also be used (see p. 828).

Protein. The total protein of normal cerebrospinal fluid varies from 15 to 40 mg. per 100 ml. The albumin averages 20 mg., globulin 6 mg. per 100 ml. Ventricular fluid contains very little protein; it may be as low as 5 mg. per 100 ml.

Albumin is increased in practically all pathologic conditions. An increase in globulin is regarded as indicating disease of the central nervous system, particularly syphilis. An increase in globulin without an increase in cells (Nonne's syndrome, albuminocytologic dissociation) may occur in case of tumor of the cord, and in infectious polyneuritis (Guillain-Barré syndrome).

Nonprotein Constituents. Methods for the determination of the nonprotein nitrogen constituents, sugar, and chlorides in spinal fluid are identical with those used for blood except that one-fourth the quantities of sulfuric acid and sodium tungstate suffices for precipitation of the protein. For estimation of sugar only, 1 ml. spinal fluid is diluted with 4 ml. water and determination made as with blood filtrate.

The nonprotein nitrogen content parallels that of the blood closely, being 15 to 35 mg. per 100 ml.; urea nitrogen 10 to 15 mg.; uric acid 1 to 2 mg.; creatinine 1 to 2 mg.

Sugar. The cerebrospinal fluid sugar is 20 to 30 mg. per 100 ml. lower than that of the blood. There appears to be a definite relationship between the cerebrospinal fluid sugar and blood sugar, with a considerable lag in the rise of the cerebrospinal fluid sugar. The average figures are 50 to 80 mg. per 100 ml. of spinal fluid, with a blood sugar of 80 to 120 mg. per ml. of blood. Any spinal fluid sugar found out of this range should be again investigated and the blood sugar determined at the same time.

Infections caused by cocci cause a reduction in the spinal fluid sugar which is proportional to the severity of the infection. Tuberculous meningitis causes a more gradual fall.

Chlorides. The chloride content of the cerebrospinal fluid, expressed as sodium chloride, averages 720 to 750 mg. per 100 ml. (124 to 128 mM). In tuberculous meningitis the chlorides fall rapidly to less than 600 mg. (104 mM). A lesser reduction (680 to 630 mg., 116 to 108 mM) occurs in acute purulent meningitis.

The pH ranges from 7.4 to 7.6. The carbon-dioxide combining power parallels that of the blood plasma (50 to 65 volumes per 100 ml.).

COLLOIDAL GOLD TEST (LANGE)

The principle of the test depends upon the facts that, if the conditions and the quantities of the reagents are properly adjusted: The addition of sodium chloride solution (an electrolyte) to a colloidal solution of gold chloride causes a precipitation of the gold chloride, with characteristic changes in the color of the solution. The colloids in normal cerebrospinal fluid exert a protective action on the colloidal gold chloride which prevents this precipitation. In many pathologic conditions the cerebrospinal fluid loses this protective action, and precipitation occurs.

Many technical difficulties are encountered in preparing a suitable colloidal gold solution. Williams' method (1935) of preparing the solution as modified by O. C. Western is now used by the U. S. Naval Medical School and has given great satisfaction.

All glassware used in the preparation and titration of the solutions, in collection of the spinal fluids, and in the tests must be Pyrex and chemically clean. It may be cleaned with sulfuric acid-bichromate cleaning solution, then rinsed thoroughly with tap water and finally with distilled water. The spinal fluid must be collected and kept sterile until tested.

Reagents

1. **Distilled Water.** Distilled water used in the preparation of the stock solution and reagents is redistilled in an all-glass Pyrex still after the addition of 10 ml. of 85 per cent phosphoric acid per liter of water. The distillate must be collected in a closed system, protected from laboratory fumes, in a large Pyrex bottle. Care must be taken that no acid is carried over into the condenser. If properly sealed it will keep at least three weeks. Further redistillation is unnecessary.

2. **Gold Chloride (Merck's Blue Label), 1 Per Cent Solution.** One or more 1-Gm. glass ampules of gold chloride are thoroughly cleaned and dried. The ampules are scratched and broken, and with their contents are dropped into a beaker. A little distilled water is added to dissolve the gold chloride, and this is poured into a volumetric flask. The beaker and contents are rinsed with several small portions of distilled water which are added to the flask; the contents of the flask are diluted to the mark and mixed.

3. **Potassium Oxalate, 1 Per Cent Solution.** Exactly 5.5409 Gm. of potassium oxalate ($K_2C_2O_4.H_2O$) (reagent grade) are weighed out, transferred quantitatively to a 500-ml. volumetric flask, and dissolved in distilled water. The solution is diluted to the mark and mixed.

4. **Potassium Hydroxide, 0.02 N Solution.** Five hundred and thirty Gm. potassium hydroxide (reagent grade) are dissolved in sufficient distilled water to make 1 liter. To this are added 5 Gm. calcium hydroxide suspended in 50 ml. distilled water. The solutions are mixed and allowed to stand in a 1-liter stoppered Pyrex cylinder until clear. A small amount is then removed and titrated against a standard acid solution. The exact amount required to make 2 liters of 0.02 N solution (approximately 5 ml.) is calculated. Just before use, the calculated volume is transferred to a volumetric flask and diluted to the mark with distilled water.

5. **Hydrochloric Acid 0.02 N.** Five hundred and forty ml. concentrated hydrochloric

acid (reagent grade) are diluted to 1 liter with distilled water. This is titrated against a standard alkali solution, and the volume required to make 2 liters of 0.02 N solution is calculated. This quantity is transferred to a volumetric flask, and diluted to the mark with distilled water.

6. Sodium Chloride, 0.4 Per Cent Solution. This is prepared in the usual manner.

Procedure for Preparing the Solution

1. Two liters of distilled water are placed in a 4-liter Erlenmeyer flask; 20 ml. of 1 per cent gold chloride solution are added and mixed by swirling.

2. Twenty ml. of 1 per cent potassium oxalate solution are added and the flask is again swirled.

3. *Immediately,* 85 ml. 0.02 N potassium hydroxide are added. The contents of the flask are mixed by swirling and heated over a large burner so that the solution boils after eight to nine minutes. The color of the solution changes to a pale blue, then a reddish-purple, then a deep dark red. At about the time the solution boils it should change (brighten) to a bright orange-red color. After this change in color takes place the solution should be boiled about one minute longer, then removed from burner, asbestos gloves being employed, swirled, and poured into a large Pyrex pooling jar. After cooling (and preferably after standing two to three weeks) the solution is ready for titration.

The final "flash" to a bright orange-red color is essential for a satisfactory solution. If this does not occur the fault is probably in the amount of alkali added and the process should be repeated, adding 1 to 2 ml. more (or less) of 0.02 N potassium hydroxide, until an amount is found which gives the proper change in color. This volume (instead of 85 ml.) should be added whenever this lot of potassium hydroxide is subsequently used in making gold chloride solution.

Standardization of the Solution

1. If a spinal fluid is available which gives a positive (paretic type) reaction with a gold chloride solution known to be satisfactory, 5 ml. of this fluid are diluted to 50 ml. in a volumetric flask with 0.4 per cent salt solution. A second dilution is prepared by transferring exactly 25 ml. from this flask to a second 50-ml. volumetric flask, and diluted to the mark with 0.4 per cent salt solution. This process is continued until 10 dilutions have been prepared (1 : 10 to 1 : 5120, inclusive).

Table 70

DILUTION OF SPINAL FLUID

	Tube 1	*Tube 2*	*Tube 3*	*Tube 4*	*Tube 5*	*Tube 6*	*Tube 7*	*Tube 8*	*Tube 9*	*Tube 10*	*Series*
Amount of acid per 30 ml. gold solution											
0.3 ml.	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	1
0.4 ml.	"	"	"	"	"	"	"	"	"	"	2
0.5 ml.	"	"	"	"	"	"	"	"	"	"	3
0.6 ml.	"	"	"	"	"	"	"	"	"	"	4
0.7 ml.	"	"	"	"	"	"	"	"	"	"	5
0.8 ml.	"	"	"	"	"	"	"	"	"	"	6
0.9 ml.	"	"	"	"	"	"	"	"	"	"	7
1.0 ml.	"	"	"	"	"	"	"	"	"	"	8
Reference gold solution	"	"	"	"	"	"	"	"	"	"	9

If a positively reacting spinal fluid is not available a 1 : 400 dilution of normal human serum in distilled water is substituted for the spinal fluid.

2. Thirty ml. of the colloidal gold solution to be tested are transferred into each of eight glass-stoppered 50-ml. Erlenmeyer flasks. To each flask is added 0.02 N hydrochloric acid in increasing amounts: 0.3 ml. to the first, 0.4 ml. to the second, increasing the amount added to each successive flask by 0.1 ml. until 1.0 ml. is added to the eighth flask. If a gold chloride solution of known reliability is available, 30 ml. of this are placed in a ninth flask and the necessary amount of 0.2 N hydrochloric acid shown by previous titrations of that solution is added.

Procedure

1. In suitable racks, set up eight series of 10 test tubes each for the solution to be tested (150-mm. by 16-mm. Pyrex tubes) and a ninth series of 10 tubes for the reference gold solution.

2. Into each tube pipet 0.5 ml. of diluted spinal fluid as indicated in Table 70, the tubes in each vertical column receiving the dilution indicated at the top of the column.

3. Pipet into each tube 2.5 ml. gold chloride solution, the tubes in each horizontal column receiving solution acidified with the volume of 0.02 N hydrochloric acid indicated in the column at the left.

4. The contents of each tube are mixed by holding the tube in the hand and gently striking the side of the tube with the other hand. The tubes are covered with a clean towel and allowed to stand overnight.

5. The results obtained are compared with the series containing the reference gold solution. The amount of acid added to the series of tubes matching this is to be used in subsequent tests. If no reference solution is available, the series showing complete precipitation in the first four tubes but not in more than five is selected.

6. In another series of 10 tubes is placed 0.5 ml. of similar dilutions of a normal spinal fluid, and to each are added 2.5 ml. of gold chloride solution acidified with the volume of acid found optimal in the titration above. The contents of these tubes are mixed and allowed to stand overnight. If the reaction is negative, the solutions are suitable for use. The solutions must be stored in tightly stoppered Pyrex bottles, preferably in a dark cool place. According to Western, such solutions have been found not noticeably changed after five years' storage.

Actual Test on Spinal Fluids

The following modification of Lange's Colloidal Gold Test has proved equal to the standard technic both in correctness and legibility, and is recommended in the interest of economy.

1. Eleven chemically clean test tubes are placed in a rack.

2. Into the first tube are placed 1.8 ml. of 0.4 per cent sodium chloride solution and 0.5 ml. is placed into each of the remaining tubes.

3. To the first tube is added 0.2 ml. of spinal fluid and the contents are mixed thoroughly. (We feel that the dilution will be more accurate if 0.2 ml. of fluid is diluted with 1.8 ml. of salt solution than if 0.1 ml. is diluted with 0.9 ml. of salt solution. If the latter is used, 1.0 ml. is not discarded from the first tube as directed in Step 4.)

4. One ml. is discarded from the first tube, then 0.5 ml. is transferred to the second tube and mixed thoroughly. Five-tenths ml. is transferred to the third tube, and this procedure is continued until the tenth tube is reached; from this tube 0.5 ml. is discarded. The eleventh tube is used as a control.

5. To each tube 2.5 ml. of colloidal gold solution are added.

6. The contents of the tubes are mixed thoroughly and the tubes set aside for 24 hours. The readings are made and recorded as in the original test.

The results are usually expressed numerically as follows:

Unchanged deep red	0
Bluish red	1
Lilac or purplish	2
Deep blue	3
Pale blue	4
Colorless	5

With a normal cerebrospinal fluid there is no change in color in any of the tubes.

Color change of slight degree occurring in the first two or three tubes alone is not significant. The tube used for collecting the fluid should be cleaned in the same way as those used in the colloidal gold test.

Three types of reaction occur:

1. *Paretic type* (first or left zone), in which the maximum change (complete precipitation) occurs in the first four or five tubes; e.g., 5555432100.

2. *Luetic type* (second or mid-zone), in which the maximum change (usually not complete precipitation) occurs in about the fourth and fifth tubes; e.g., 0124321000.

3. *Meningitic type* (third or right zone), in which the greatest change is in about the seventh and eighth tubes; e.g., 0001234421.

A minute trace of blood in a normal spinal fluid may give a meningitic curve. Any appreciable amount of blood renders the test entirely unreliable, as does even a slight degree of bacterial contamination.

The chief practical value of the test is in the diagnosis of syphilis of the central nervous system, and particularly in differentiating general paresis from other types of syphilis. A diagnosis of paresis should not be based on a paretic curve alone, but the latter always indicates a grave type of infection which demands energetic treatment. However, a paretic curve (with a negative Wassermann reaction) occurs in some cases of multiple sclerosis.

Colloidal Mastic Test. This test is useful to supplement the colloidal gold test, and it is a fairly satisfactory substitute for the gold test if circumstances make it impracticable to perform the latter. The mastic test has the following technical advantages: The mastic solution is easy to prepare (although not every lot is satisfactory). Ordinary distilled water suffices, and extraordinary precautions in cleaning apparatus are not necessary. A trace of blood or a slight degree of bacterial contamination does not interfere seriously, as it does with the colloidal gold test. The mastic test fails, however, to yield the different types of curves given by the colloidal gold test.

Mastic Solution. Ten Gm. gum mastic are dissolved in 100 ml. of absolute alcohol and the mixture is filtered repeatedly until an opalescent solution free from turbidity is obtained. Just before use 1.0 ml. is diluted to 10 ml. with absolute alcohol and this is poured into 40 ml. of (ordinary) distilled water.

Diluting Solution. To 990 ml. of a 1.25 per cent solution of sodium chloride are added 10 ml. of a 0.5 per cent solution of potassium carbonate.

Test. (1) A series of 10 small test tubes is set up; to the first tube are added 1.5 ml., and to the others 1.0 ml. of diluting fluid. (2) To the first tube is added 0.5 ml. of spinal fluid; the contents of the tube are mixed and 1.0 ml. is transferred to the second tube. After mixing, 1.0 ml. is transferred to the third tube, and this process is continued until

the ninth tube is reached, 1.0 ml. being discarded from this tube. The tenth tube serves as a control. (3) To each tube is added 1.0 ml. of freshly diluted mastic solution. The tubes are shaken and allowed to stand 12 hours or more at room temperature. They are then read.

The control tube is opalescent but remains clear. Five degrees of reaction are noted: milky turbidity, 1; cloudy with minute flocculi, 2; cloudy with coarser flocculi, 3; a coarse flocculent precipitate with a cloudy fluid, 4; and complete precipitation, 5.

A reaction of $+^1$ or $+^2$ in the first two or three tubes is of no significance. A $+^3$ reaction is suggestive, and a $+^4$ or $+^5$ is definitely pathologic. The maximum reaction practically always occurs in the first few tubes; the reaction is (qualitatively) "paretic" in type, regardless of the type of cerebrospinal syphilis present. Otherwise its significance is the same as that of the colloidal gold reaction. It is about equal to the latter in sensitiveness.

Cerebrospinal Fluid Changes in Various Diseases

Acute Anterior Poliomyelitis. Early in this disease the cerebrospinal fluid will be clear with a moderate increase in cells (20 to 30, of which 90 per cent or more are lymphocytes). After several hours a second puncture may yield fluid containing 50 per cent or more of polymorphonuclear leukocytes, while a subsequent puncture reveals a shift to lymphocytes again. This cellular shift from polymorphonuclear leukocytes to lymphocytes, when found, definitely suggests anterior poliomyelitis. There are no other abnormal findings.

Acute Benign Lymphocytic Choriomeningitis. In this disease the cerebrospinal fluid is under increased pressure with an increase in lymphocytes. There are no other pathologic findings.

Alcoholism. In acute alcoholism the cerebrospinal fluid will be found under increased pressure and alcohol will be found in the fluid. (See Determination of Alcohol in Blood, p. 792.)

Diabetes Mellitus. In *coma* from diabetes the cerebrospinal fluid shows an increase in sugar, no increase in cells, and a pressure which is lower than normal.

Epidemic Cerebrospinal Meningitis. In this condition the pressure is increased; the fluid is cloudy; there is an increase in cells, 85 per cent of which are polymorphonuclear leukocytes; and Gram-negative cocci (*Neisseria intracellularis*) are present.

Epidemic Encephalitis. During the acute stage there is often a moderate increase in globulin and in cells which are all lymphocytes. In chronic cases with the Parkinsonian syndrome the fluid is normal.

Lead Poisoning. The cerebrospinal fluid shows an increase in pressure and an increase in lymphocytes, and the dithizone test may show definite evidence of lead. There are signs of hypertensive encephalopathy.

Multiple Sclerosis. During the active progressive periods of the disease the globulin is markedly increased, but the cells as a rule are not notably increased. There may be a paretic type of gold chloride reaction, but a negative Wassermann reaction.

Subarachnoid Hemorrhage. The fluid is under increased pressure, is grossly bloody, and after the cells have been removed by centrifugation shows a distinctly yellowish color.

Syphilis. The cerebrospinal fluid is under increased pressure; the cell count is increased (usually 25 to 100 lymphocytes); the globulin is increased; the Kahn and Wassermann reactions are positive. A colloidal gold curve in the first zone indicates paresis; a second-zone curve is found in other types of involvement of the central nervous system (tabes, syphilitic meningitis, etc.).

Tuberculous Meningitis. This disease causes an increase in pressure, an increase in lymphocytes, and a decrease in sodium chloride and usually in sugar content. This finding is practically pathognomonic.

Bacteriologic Examination

If meningitis is suspected, or if the fluid is cloudy, a portion should be inoculated immediately on blood agar (or dextrose brain broth) *and the tubes kept at body temperature until placed in the thermostat.* If the fluid is cloudy films should be made and stained by Gram's method, and if indicated, for tubercle bacilli.

In addition to the meningococcus, many other species of bacteria may cause a meningitis, most frequently pneumococci, streptococci, and staphylococci. The meningitis may be hematogenous in origin, one manifestation of a general septicemia, or it may result from direct extension from some localized area of infection, most frequently in the middle ear, mastoid, or paranasal sinuses.

If *tuberculous meningitis* is suspected and a clear or opalescent fluid is obtained, a tube of fluid may be put in the icebox (Levinson recommends room temperature), *kept undisturbed,* and examined after 24 hours. If a fine filmy clot has formed, this should be fished out carefully with a platinum needle, spread over a small area on a slide, and stained for tubercle bacilli in the usual way. The bacilli can be demonstrated without great difficulty in a large majority of the cases.

A better method is that of pipetting 0.1 ml. of 1 per cent potassium alum solution into 10 ml. of fluid, shaking for 10 minutes, and centrifuging. Smears are made of the sediment, and cultures are made and guinea pigs inoculated if necessary.

The bacilli may also be concentrated with chloroform (see p. 82) if only stained films are desired.

If no tubercle bacilli are found, a portion of the clot or sediment should be inoculated on suitable media, and a guinea pig inoculated.

If benign lymphocytic choriomeningitis is suspected, several mice should be inoculated intracerebrally, or guinea pigs subcutaneously.

Animal Parasites

Trypanosomes may be found in the spinal fluid in the advanced "sleeping-sickness" stage of trypanosomiasis. The lymphocytes are increased.

Trichinella embryos have been found in the spinal fluid.

Exudates and Transudates

The serous cavities normally contain only minute amounts of fluid. Large amounts of fluid may accumulate in them under pathologic conditions. Such fluids are divided into two classes: (1) transudates, which form as a result of circulatory stasis or obstruction, and (2) exudates, which result from inflammatory processes. It is practically important and often possible to differentiate between the two conditions by a study of the characteristics of the fluid.

If possible several ounces of fluid should be collected in sterile condition, and a portion should be mixed with a little potassium oxalate or 2 per cent sodium citrate solution to prevent clotting. The following examinations should be carried out:

1. **Gross Appearance.** Transudates are clear and transparent or opalescent, and pale yellowish in color. Exudates are usually more highly colored and more turbid. They may vary from serous (slightly cloudy) or serofibrinous to purulent; or they may be hemorrhagic or chyliform.

A trace of blood is common as a result of trauma of the puncture. A *hemorrhagic fluid* is frequently obtained in cases of malignant disease, but may occur also in acute tuberculous infections, and in a variety of other conditions (trauma, hemorrhagic diseases, aneurysms, etc.).

A true *chylous fluid* (from which the fat can be extracted with ether) results from

Table

CEREBROSPINAL FLUID IN
(After Fremont-Smith and Ayer in *Jour*

Disease	*Initial pressure: mm. of Spinal Fluid Horizontal Position*	*Rise on Jugular Compression*	*Appearance*	*Cells per cu. mm.*	*Globulin mg./100 ml.*
Lumbar Cisternal Ventricular	70–190* .. (Water manometer)	.. Prompt ..	Clear Colorless No clot	0–5 0–5 0–5	.. .. ..
Blood plasma	..	..	..	..	..
Brain abscess	+	N or delayed in some cases	Clear and colorless to turbid; ± clot	+	±
Acute purulent meningitis (Coccal infection)	+	±	Purulent; faint yellow; ± clot	+ (Polys)	+
Acute anterior poliomyelitis	+	N	Slightly opalescent; rarely turbid; faint yellow; ± delicate fibrin web	Polys early shifting to lymphocytes	Slightly +
Benign lymphocytic choriomeningitis (Aseptic lymphocytic meningitis)	+	N	Clear to hazy	+ (Lymphs)	±
Tuberculous meningitis	+	N	Opalescent to turbid; faint yellow; ± delicate fibrin web	± Lymphocytes	+

N—Normal.

+—Plus (positive)

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DIFFERENTIAL DIAGNOSIS
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Protein, mg./100 ml.	*Sugar, mg./100 ml.*	*Chlorides (as NaCl), mg./100 ml.*	*Non-protein Nitrogen, mg./100 ml.*	*Gold Sol.*	*Comment*
15–40 10–25 5–15	50–75† 50–75† 50–75†	720–750	12–18	0000000000	Sugar and chloride values apply to fasting nonfebrile individuals
6200–8200	70–110	570–620	18–30		
Slight increase	N or +	N	N	Variable	Polys nearly always present. If complicated by septicemia or high fever chlorides may fall to low levels
+	Low	±	N	Variable	Sugar may be but slightly decreased at outset. Often falls rapidly to under 10 mg. Chlorides rarely below 640 unless septicemia is present. Meningococci found in smear and culture with difficulty; pneumococci, streptococci, and staphylococci easily
Slight +	N	N or slight decrease	N	Variable	In preparalytic stage polys may exceed 80%—Rapid change to lymphocytes. With gradual increase in cells, protein increases for 2 to 3 weeks. Paralysis rarely present until marked lymphocyte increase (90%)
±	N	N	N	Variable	Signs of meningeal irritation with increase of lymphocytes; culture sterile, and animal inoculation negative for tuberculosis. Disease benign in nature. Etiology: filtrable virus. Often mistaken for tuberculous meningitis
+	N or low	Very low chlorides almost diagnostic	N	Variable	Excess fluid. Polys apt to be increased very early and in infants. Chlorides usually below 640 mg./100 ml. Tubercle bacilli found in clot or sediment. Guinea-pig inoculation positive

±—Plus-minus normal or increased.

*Divide by 13.5 to convert to mm. of mercury.

†Depends upon blood sugar level.

Table 71

CEREBROSPINAL FLUID IN
(After Fremont-Smith and Ayer in *Jour*

Disease	*Initial pressure: mm. of Spinal fluid Horizontal Position*	*Rise on Jugular Compression*	*Appearance*	*Cells per cu. mm.*	*Globulin mg./100 ml.*
Encephalitis Economo type A Encephalitis Japanese type B Encephalitis St. Louis type Equine encephalomyelitis	N	N	N	N or slight increase No polys	±
Acute syphilitic meningitis	+	N	Clear to turbid; faint yellow; ± fibrin clot	+	+
"Meningovascular syphilis"	±	N rarely delayed	N rare fibrin clot with large fiber	+	+
Progressive parenchymatous syphilis	+	N	N rare fibrin clot	+	+
Late inactive forms or insufficiently treated	N	N	N	N	N
Alcoholism	+	N	Clear	+ (Lymphs)	N
Diabetes (coma)	Low	N	Clear	+ Lymph	N

N—Normal.
+—Plus (positive).

obstruction to large lymphatic channels (filariasis, occasionally cancer or tuberculosis of the lymph nodes, etc.).

2. Specific Gravity. This may be determined with an ordinary urinometer. Transudates are under 1.018 and usually under 1.015; exudates are usually over 1.018.

3. Protein Content. The amount of protein in the fluid may be determined by means of the Esbach tube, as described for urine, first diluting the fluid 1 : 10 with water and acidifying; or the sulfosalicylic acid method may be used. Transudates contain less than 30 Gm. (and usually less than 25 Gm.) per liter; exudates, usually 30 Gm. or more. Transudates do not clot. Exudates contain fibrinogen and often clot on standing. Exudates differ also in containing "seromucin" ("nucleo-albumin").

RIVALTA'S TEST FOR SEROMUCIN. To 100 ml. water in a graduate, 2 drops of glacial acetic acid are added. A drop of the fluid to be tested is allowed to fall into the dilute acid. A positive reaction is indicated by the appearance of a bluish-white cloud.

—(*Continued*)

DIFFERENTIAL DIAGNOSIS
nal of American Medical Association)

Protein, mg./100 ml.	*Sugar, mg./100 ml.*	*Chlorides (as NaCl), mg./100 ml.*	*Non-protein Nitrogen, mg./100 ml.*	*Gold Sol.*	*Comment*
N or slight increase	N	N	N	Variable	Sugar is normal unless blood sugar is elevated. Over 50% of patients have normal cell count. Others rarely exceed 60 cells. Protein increase when present is slight—rarely reaching 100 mg./100 ml.
+	N	Slightly low	N	Strong reaction. Zone variable	Wassermann reaction nearly always positive
+	N	N	N	Variable	Wassermann reaction nearly always positive
+	N	N	N	Paretic or luetic	Wassermann reaction always strongly positive (includes tabes 80%, paresis 100%, and optic atrophy 80%)
±	N	N	N	Weak luetic ±	Wassermann reaction weakly positive or negative
N	N	N	N	N	Alcohol present in the spinal fluid
N	Marked increase	N	N	N	Increased sugar in sp. fl.

±—Plus-minus normal or increased.

*Divide by 13.5 to convert to mm. of mercury.

†Depends upon blood sugar level.

Moritz adds 2 drops of 5 per cent acetic acid to the fluid. A heavy cloudlike precipitate indicates an exudate. A transudate may show a slight opalescence.

4. Cell Count. The *total cell count* is made with a hemacytometer. In the case of serous fluids with relatively few cells, the fluid is used undiluted or mixed with a little staining solution, as described for spinal fluid. Cloudy or purulent exudates should be diluted in a leukocyte pipet as in making a blood count.

To make a *differential count,* a portion of the fluid is centrifuged *as soon as possible* after it is secured, the fluid is decanted, the sediment is resuspended in the drop remaining in the tube, and *thin* films are made, using the same technic as in making blood films. Films may be stained by Giemsa's or Wright's stain, or they may be fixed by heat (flaming alcohol) and stained with hematoxylin and eosin. The cells degenerate rapidly if the fluid is allowed to stand and then stain poorly. In purulent exudates the leukocytes often show marked toxic-degenerative changes. If the films are thick and the cells rounded

up and not well spread out, it is difficult or impossible to identify them. The interpretation of such films is often difficult and requires considerable experience. It is impossible to make satisfactory counts or films from clotted fluid.

In *transudates* the cell count is low, usually under 100, and nearly all the cells are large mononuclear mesothelial cells. These may be in plaques. A few lymphocytes may be present.

In *tuberculous exudates* the cell count is moderately high, usually from 100 to 1000 or more, and nearly all the cells are lymphocytes. A few large mononuclear cells may be present, and in acute cases some leukocytes.

In *exudates due to other organisms* the cell count is much higher, often many thousands, and nearly all are polymorphonuclear leukocytes.

A serous exudate may develop without actual infection of the cavity as a response to some localized infection in the immediate vicinity (e.g., a lobar pneumonia or a perirenal abscess). Such fluids show a more moderate increase in cells which are largely leukocytes, and yield sterile cultures.

Exudates due to malignant disease may contain tumor cells, but it is difficult to identify them with certainty. The most important points are the occurrence of clumps of cells, and the presence of mitotic figures. These are best demonstrated by fixing the moist films with some bichloride fixative and staining with hematoxylin and eosin. In some cases there are very large, vacuolated cells which have the appearance of a signet ring, but similar cells have been reported in chronic inflammatory processes.

More satisfactory results have been reported with Mandelbaum's method. The sediment from a considerable amount of fluid is collected by sedimentation and centrifugation. To the sediment in the tip of the centrifuge tube, 10 per cent formalin is added and allowed to remain for 24 hours. The sediment is then dehydrated, embedded in paraffin; sections are made and stained with hematoxylin and eosin as is done in examining a piece of tissue.

The *differences between transudates and exudates* are not always clear-cut. A transudate may acquire the characteristics of an exudate after it has become chronic, particularly after repeated tappings. It may become concentrated and show a high specific gravity and high protein content during the process of absorption. An exudate due to a mild chronic infection, particularly in the peritoneal cavity, may show a relatively low gravity and protein content, like a transudate. Stasis may play a part in the accumulation of such fluids.

5. Bacteriologic Examination. Cultures should be made on blood agar. If the fluid is cloudy or purulent, films should be stained by Gram's method, and with methylene blue.

In cases of *acute peritonitis* Wilkie pointed out that the examination of stained films of the exudate gives information of prognostic value. If bacteria are sparse and largely intracellular, the outlook is good. If they are numerous and largely extracellular, and if the leukocytes show degenerative changes, the prognosis is unfavorable.

If *tuberculosis* is suspected, cultures of the centrifuged sediment should be made and guinea pigs inoculated. Positive results may be expected in at least 50 per cent of the cases from pleural fluids, less frequently from ascitic fluids. A portion of the sediment may be stained for acid-fast bacilli, but they are found only occasionally.

Synovial Fluid. This resembles the fluid from the serous cavities. The normal cell

count is about 50, and 95 per cent of the cells are mononuclears. The sugar and pH are practically the same as in the blood.

In *chronic infectious* (*rheumatoid*) *arthritis* cultures of the fluid are sterile. The cell count is increased, but usually under 5000, and less than 50 per cent of the cells are polymorphonuclear leukocytes. The sugar and pH are slightly reduced (60 or less, about 7.0).

In *acute infectious arthritis* from which positive cultures are obtained the cell count is usually over 10,000, and over 50 per cent are polymorphonuclear leukocytes. The sugar is under 45 mg. and the pH under 7.0.

Traumatic arthritis is suggested by the presence of red cells in the fluid and by an icterus index over 6.

In *tuberculous arthritis* with effusion organisms can practically always be demonstrated by culture or guinea-pig inoculation.

CHAPTER 36

Chemical Examinations of the Blood

For practical clinical purposes, to avoid wasting time and effort, it is important to select those procedures which are likely to yield useful information in the disorder which is present or suspected, and not to follow blindly some fixed routine analytical procedure. Tests which are indicated should be done accurately and preferably in duplicate. An accurate interpretation of the results can be made only in conjunction with all the other available information concerning the patient. In general these analyses are of less value as direct diagnostic procedures than as a means of recognizing disorders of metabolism and of guiding therapeutic measures necessary to relieve them.

Table 72 gives a summary of the normal figures and alterations which may be expected in various clinical conditions. The figures for average normal findings are taken from Peters with a few unimportant exceptions. The others have been compiled from various sources. The values are given in mg. per 100 ml. of whole blood (the usual system) or as millimoles (mM) or milliequivalents (mE) per liter, unless otherwise indicated. "Inc." and "Dec." signify increased and decreased, respectively; "N" signifies normal; "Var.," variable. "To" qualifying a figure indicates that findings range from normal to that figure. Workers should notice the marked differences between the concentration of many of these substances in cells and in plasma and the effect that anemia alone may have on the results of analyses if they are made with whole blood.

Collection of Blood. To secure uniform conditions the blood should be obtained, usually by venepuncture, before breakfast in the morning. This is essential for determination of sugar and inorganic phosphorus. For most other substances fairly comparable results may be obtained if blood is taken four hours after a meal. Many determinations may be made on whole blood, and coagulation is prevented by adding lithium oxalate or potassium oxalate (not ammonium oxalate), using preferably 2 mg. (not over 3 mg.) per ml. of blood. *For determination of calcium, serum is used and anticoagulants (particularly oxalate) must be avoided.* Serum (or plasma) is required for determination of proteins and inorganic phosphorus, and is decidedly preferable for uric acid, chloride, cholesterol, carbon-dioxide combining capacity, and bases. It is equally satisfactory for all the other usual analyses (except hemoglobin and O), although a larger volume of blood is required.

Serum is preferable to plasma, since the presence of oxalate may interfere in some of the tests. Furthermore, the use of anticoagulants increases the tendency to hemolysis. Tingeing of the serum with hemoglobin causes errors in many of the tests and must be avoided. All glassware used, *including the syringe and needle,* must be chemically clean and should be dry. Preferably the latter should be wrapped, sterilized in a steam sterilizer, and dried in an oven. If boiling must be employed for sterilization, the implements should be boiled in distilled water (not tap water) and dried in the air. For most determinations (except sodium and chloride), however, they may be rinsed in sterile physiological sodium chloride solution.

The following procedure (Peters and Van Slyke) is recommended for the *preparation of the oxalate tubes.* A 20 per cent solution of potassium oxalate is prepared. If alkaline, it should be recrystallized, and if necessary enough oxalic acid added to bring the reaction

to a pH of 7.4 (to phenol red, see p. 924). To a series of tubes, with the aid of a graduated pipet, 0.05 ml. of the solution (for 5-ml. samples) and 0.1 ml. (for 10-ml. samples) are added. Each tube is held horizontally, rotated until a thin film of oxalate solution is distributed over the walls, and dried without heating in a current of air.

For determination of sugar and nitrogenous substances, blood may be taken in any convenient way and mixed with a little potassium oxalate in open bottles. For determination of blood gases, disturbances of acid-base equilibrium, and for accurate studies of chlorides and other electrolytes the blood must be protected from exposure to air by collecting it under oil. We recommend the apparatus shown on p. 928. Stasis must be avoided, and if a tourniquet is required, one should loosen it after the needle has entered the vein and wait until normal conditions are restored before *gently* aspirating the blood.

After blood has been obtained the protein precipitation or other analytical procedures should be carried out without delay. If this is not possible, the blood should at once be chilled in ice water, and kept cold till used. In a hot laboratory the glucose content falls quite rapidly (after one to two hours), with simultaneous formation of lactic acid, owing to the action of a glycolytic ferment which is eliminated with the proteins. Other ferments (phosphatase) break down organic phosphate and increase the amount of inorganic phosphorus present. Most other determinations (except acid, pH, and CO_2) show less change during the first six or eight hours, but the longer the analysis is delayed, the greater are the errors encountered. Blood can be preserved two or three days at ordinary temperatures for glucose and nonprotein-nitrogen determinations, if it is sterile and if 10 mg. sodium fluoride and 1 mg. thymol per ml. are added to prevent coagulation and inhibit ferment activity.

In the selection of the following procedures, consideration has been given to simplicity of technic and stability of the reagents as well as to the precision of the results obtainable. We believe that if carefully carried out, they will give results amply accurate for all clinical purposes.

For the determination of glucose and the nonprotein nitrogenous constituents of the blood the *Folin-Wu system* of analysis is satisfactory, and the following procedures utilize their tungstic acid filtrate and largely follow their technic.

Precipitation of Protein (Folin-Wu, Haden). (1) In a clean dry flask pipet (preferably with a Folin pipet) one volume of well-mixed blood (10 ml. for a complete analysis). (2) From a graduate or buret add slowly with constant stirring 8 volumes of N/12 sulfuric acid. (3) Add 1 volume of 10 per cent sodium tungstate solution; shake thoroughly. If the precipitation is complete the color becomes chocolate-brown; on shaking, it gives a ringing metallic sound; and it shows practically no foam. (4) After 10 to 20 minutes filter through a dry ammonia-free filter large enough to hold the entire quantity. Refilter the first few drops if necessary. The filtrate must be perfectly clear and colorless. It should be acid to litmus but not to Congo red. If brownish or if it foams appreciably on shaking, add one drop of 10 per cent sulfuric acid, shake for a few minutes, and continue until a clear, faintly acid filtrate is obtained. The filtrate may be kept for 24 hours in the icebox, with a drop of xylol as preservative.

For plasma or serum, dilute both the N/12 sulfuric acid and sodium tungstate solution with an equal volume of water, and proceed as above, using the diluted reagents.

N/12 Sulfuric Acid. Add 2.5 ml. concentrated sulfuric acid to 1 liter water. Check the strength of the solution with an accurately standardized alkali solution (see p. 921).

Sodium Tungstate Reagent. Dissolve 100 Gm. pure sodium tungstate in 1 liter ammonia-free water. Filter if necessary. The reaction should be faintly alkaline, but such that less than 0.4 ml. N/10 hydrochloric acid will render 10 ml. neutral to phenolphthalein. If necessary add acid or alkali sufficient to bring it to this point.

Check both solutions by showing that they yield a suitable filtrate with normal blood. If tightly stoppered they keep indefinitely.

Table 72

QUANTITIES OF SOME IMPORTANT CONSTITUENTS OF THE BLOOD IN HEALTH AND DISEASE

	Non-protein Nitrogen	*Urea Nitrogen*	*Uric Acid*	*Creatinine*	*Glucose (True)*	*Sodium*	*Potassium*	*Calcium*	*Inorganic Phosphorus (Plasma)*	*Chloride as Cl*
Normal:										
Whole blood	39	18	3	1.2	86*	172	197	5.6		285
Range	25–39	12–18	2–4	1.2	60–100					
Plasma or serum	29	19	3	1.2	96	310	18	10	3	362
Range	..	..	..	..	..	140–145 mM	5 mM	9–11.5 5 mE	3–4.5 ..	352–380 99–108 mM
Cells	49	17	3	1.2	75	Trace	420	0		192
Anemia (50%)	33	18	3	1.2	91	241	107	7.8		323
Diabetes moderate	..	..	..	..	150–300					
Diabetic coma	..	15–30	..	1–4	300–1000	..	..	..	..	Dec.
Pancreatic disease	..	..	..	..	Inc.					
Arteriosclerosis	..	..	3–5	..	N-Inc.	..	..	N	N	N
Acute nephritis	40–250	20–200	5–15	2–10	Inc.	..	..	..	..	N-Inc.
"Nephrosis"	N-Inc.	N-Inc.	..	1–4	N-Inc.	Var.	N	Dec.	N-Inc.	N-Inc. To 120 mM
Contracted kidney	90–350	To 250	To 10	To 16	Inc.	..	..	N-Dec.	To 20	Var.
Uremia	90–350	To 300	Inc.	Inc.	Inc.	To 128 mM	N	Dec.	To 20	Var.
Bichloride poisoning	To 350	To 300	To 16	To 3.5	Inc.	..	..	..	..	Dec.
Gout	..	..	4–10							
Hyperthyroidism	N-Inc.	N-Inc.	..	..	Inc.	..	..	N-Dec.		
Myxedema	..	..	..	..	Dec.	..	..	N-Inc.		N-Dec.
Hyperparathyroidism	N-Inc.	N-Inc.	..	..	..	..	..	To 18	Dec.	
Hypoparathyroidism	..	..	..	..	..	..	..	To 7	Inc.	
Addison's disease	Inc.	Inc.	..	..	Dec.	Dec.	Inc.	..	..	Dec.
Pneumonia	To 70	N-Inc.	N-Inc.	..	..	Dec.	..	..	..	To 80 mM
Eclampsia	To 45	To 25	Inc.	To 2.5	N-Inc.	Dec.	..	N	..	N
Necrosis of liver	Inc.	Dec.	..	..	Dec.					
Intestinal obstruction	To 300	Inc.	N	N	..	Dec.	..	Dec.	Inc.	Dec.
Pernicious anemia	..	..	..	..	..	..	..	N	N	N
Obstructive jaundice	..	..	..	..	N-Inc.	..	..	N-Dec.	N	

Table 72—(Continued)

	Cholesterol	*Fatty Acids*	*Bilirubin (Plasma)*	*Diastase (Units)*	*CO_2— Vol. % (Bicarbonate)*	*Total Protein, %*	*Albumin, % (Plasma)*	*Globulin, % (Plasma)*	*Hemoglobin, Gm., %*
Normal:									
Whole blood	140–210	290–410	..	..	58	19.5	..	..	15.6
Plasma or serum	207	355	0.1–0.4	8–64	68	7.0	5.0	2.0	0
Range	140–210	290–420	..	..	50–75 22–33 mM	6.3–8.0	3.8–5.2	2.0–3.5	
Cells	..	..	..	..	45	35	..	..	35
Anemia (50%)	..	..	..	..	63	15.2	..	..	7.8
Diabetes moderate	Inc.								
Diabetic coma	To 800	Inc.	..	Inc.	To 10				
Pancreatic disease	..	..	..	Inc.					
Arteriosclerosis	N								
Acute nephritis	Inc.	..	..	..	N-Dec.				
"Nephrosis"	To 900	Inc.	..	..	N-Dec.	To 3.5	To 1.0	Var.	Dec.
Contracted kidney	Var.	Inc.	..	Inc.	N-Dec.	..	..	..	Dec.
Uremia	N-Dec.	Inc.	..	..	To 12	..	..	..	Dec.
Bichloride poisoning	N-Inc.	..	..	..	To 25				
Gout									
Hyperthyroidism	To 80	Dec.							
Myxedema	To 1350	Inc.	..	Dec.	..	..	..	..	Dec.
Hyperparathyroidism									
Hypoparathyroidism									
Addison's disease									
Pneumonia	Dec.	..	..	..	..	..	..	Inc.	
Eclampsia	..	..	..	..	To 30	..	..	Dec.	
Necrosis of liver	N-Dec.	..	Inc.	..	..	..	..	N-Dec.	
Intestinal obstruction	N-Inc.	..	..	..	To 120	To 11	..	..	Inc.
Pernicious anemia	To 70	Inc.	Inc.	..	..	..	..	..	Dec.
Obstructive jaundice	Inc.	..	Inc.	..	..	..	..	..	..

Nonprotein Nitrogen and Urea Nitrogen

Nonprotein Nitrogen (Folin-Wu). All reagents must of the highest purity, and free from nitrogen. Ammonia-free water must be used for all these determinations. Other tests requiring the use of ammoniacal solutions should not be carried out in the same room in which nesslerization procedures are performed.

(1) With a volumetric pipet put 5 ml. blood filtrate in a dry Pyrex ignition test tube (25 × 200 or 250 mm.) graduated at 35 and 50 ml. (The use of a wet tube causes trouble-some bumping.) (2) Add 1 ml. diluted acid digestion mixture and a dry quartz pebble. (3) Boil vigorously over a microburner, shaking constantly, until *dense* white fumes fill the tube. (4) Quickly transfer the tube to a stand, cover mouth of tube with a watch glass, and continue heating just enough to maintain visible boiling for two minutes, or until solution is clear. (5) Let cool in place for 90 seconds (not longer). (6) Add cautiously about 25 ml. water, at first a few drops at a time. (7) Cool to room temperature, and fill to the 35-ml. mark with water. (8) In a 50-ml. volumetric flask put 3 ml. of the working nitrogen standard solution, containing 0.15 mg. nitrogen, 1 ml. diluted acid digestion mixture, and about 25 ml. water. (9) To each, 1 or 2 drops gum ghatti solution may be added. (10) Nesslerize both solutions at the same time, adding to each 15 ml. Nessler's solution, shaking constantly while adding the solution; dilute the standard to the 50-ml. mark, and mix. (11) Stopper the tube containing the unknown with a clean rubber stopper, mix, and if it shows a *whitish* turbidity, due to etching of the glass, centrifuge until clear. (12) Compare in the colorimeter, reading the standard against itself.

Calculation:

$$\frac{\text{Reading of standard}}{\text{Reading of unknown}} \times 30 = \text{mg. NPN per 100 ml. of blood}$$

If the sediment after centrifugation is reddish, if the solution is not perfectly clear, or if either solution clouds before the reading is completed, the nesslerization is a failure and the test must be repeated. Clouding can often be prevented by adding 1 or 2 drops of gum ghatti solution to each tube before nesslerization. If the color of the unknown is much darker than that of the standard, the test must be repeated, using less of the filtrate.

Clouding of the solution after nesslerization may be due to improperly prepared solutions, especially an improper amount of alkali in the Nessler's solution. Check: 20 ml. N/1 HCl should be neutralized (to phenolphthalein) by from 11 to 11.5 ml. of Nessler's solution. It may also be due to the following technical errors: (1) Inadequate heating, covering the mouth of the tube too soon, or stopping before the solution is entirely clear. (2) Overheating, by boiling too long or too vigorously after covering the tube. (3) By diluting with water too soon, or more often, too long after heating is completed. (Considerable sputtering is to be expected when the first drops of water are added.) (4) By not keeping the solutions well mixed while the Nessler's is being added. (5) By waiting too long before making the readings.

REAGENTS: ACID DIGESTION MIXTURE. Mix 300 ml. 85 per cent phosphoric acid with 100 ml. concentrated nitrogen-free sulfuric acid, and 50 ml. 5 per cent copper sulfate solution; put in a tall cylinder, seal tightly to exclude ammonia, and let stand several weeks till the precipitate of $CaSO_4$ has settled out. Decant or siphon off the clear fluid.

DILUTED ACID DIGESTION MIXTURE (WORKING SOLUTION). To 1 volume of the clear solution above, add an equal volume of water.

NESSLER'S SOLUTION (KOCH-MC MEEKIN). Dissolve 30 Gm. potassium iodide in 20 ml. water, and add 22.5 Gm. iodine. Shake until dissolved, and pour out and reserve 0.5 ml. Add 30 Gm. pure metallic mercury, and shake until the solution becomes pale yellow, cooling under the tap when it gets warm. Remove a drop and test for free iodine with

starch solution. If negative, add, drop by drop, the iodine solution which had been set aside, until a positive reaction for iodine is obtained. Decant the supernatant fluid, wash the residue several times with water adding the washings to this fluid, and dilute the whole to 200 ml. Mix and add the entire quantity to 975 ml. of accurately prepared 2.5 N (10 per cent) carbon dioxide-free sodium hydroxide. Mix and let stand two weeks until clear.

2.5 N SODIUM HYDROXIDE SOLUTION. Dissolve 200 Gm. sodium hydroxide (sticks or pellets) in 200 ml. water, shaking until dissolved to prevent caking. Store in a tall, stoppered cylinder for two weeks, until carbonate has settled. Decant or siphon off the clear fluid, and dilute 187.5 ml. to 1200 ml. with distilled water. Mix, and cool. Check strength by titration against a standard solution of hydrochloric acid of known accuracy, and adjust if necessary. (1 ml. 2.5 N NaOH is neutralized by 25 ml. N/10 HCl.)

STANDARD NITROGEN SOLUTION (STOCK). Dissolve exactly 4.716 Gm. dry ammonium sulfate, of the highest purity and pyridine-free, in 1 liter water. This contains 1 Gm. of nitrogen, hence 1 mg. per 1 ml. (For the average laboratory we advise the purchase of the specially purified pyridine-free preparation.)

STANDARD NITROGEN SOLUTION (WORKING). Dilute exactly 5 ml. of this solution to 100 ml. with water. 1 ml. contains 0.05 mg. If tightly stoppered these solutions keep indefinitely.

AMMONIA-FREE WATER. The water used should give no appreciable color with Nessler's reagent. If it does, it must be freed from ammonia by adding a little sulfuric acid, and redistilling from a glass flask. The water must be protected from exposure to ammonia-containing air during the collection, by receiving it in a flask the vent tube of which is protected by a guard tube containing a little dilute sulfuric acid.

GUM GHATTI SOLUTION. This is used to stabilize colloidal suspensions. Fill a liter cylinder with distilled water. Put 20 Gm. gum ghatti in tea ball, and suspend it just below the surface of the water, letting it extract overnight. Add 1.0 Gm. benzoic acid dissolved in 10 ml. alcohol and shake. Clear by sedimentation if necessary. The solution keeps for months on ice. Gum ghatti should not be used unless it is found to be necessary, as it lessens the intensity of the color and to some extent alters the relationship between the intensity of color and the concentration of ammonia.

PERMUTIT. This is a finely granular insoluble sodium aluminium silicate, which absorbs ammonia (with liberation of sodium) in neutral or acid solutions, and liberates the ammonia when sodium hydroxide is added. Absorption is complete only in dilute solutions, and with an excess of permutit. To free the permutit from fine dustlike particles which sediment very slowly, repeatedly shake up the permutit in a flask with distilled water, wait a moment till the coarse particles have settled, and pour off the cloudy supernatant fluid. Permutit which has been used can be reclaimed (if it has not been in contact with Nessler's solution) in the following way: Wash several times with water. Add 10 per cent sodium hydroxide, shaking occasionally for an hour or so. Wash with water several times, then with 2 per cent acetic acid, and again several times with water. Dry in air without heating. Keep in bottle sealed with wax or paraffin.

Nonprotein Nitrogen (Buell, Adapted from Gentzkow). The following procedure has been used by Dr. Mary Buell in the Chemical Laboratories of the Johns Hopkins Hospital. It is recommended because the solutions do not cloud, whereas with the original Folin-Wu procedure it is often difficult to avoid slight degrees of clouding which are particularly disturbing if a photoelectric colorimeter is used. All reagents must be of the highest purity and free from nitrogen. Ammonia-free water must be used for all these determinations.

(1) Put 2 ml. Folin-Wu filtrate in a dry Pyrex tube graduated at 20 ml. (2) Add 0.5 ml. digestion mixture and one vitreosecal boiling chip or a quartz pebble. (3) Heat over a microburner so adjusted as to bring to boiling in 30 seconds. (4) When charring begins add 5 drops of persulfate solution, 1 drop per second, without removing the flame. (5) Insert a small funnel in the tube and heat until digestion is complete. (If

boiling is stopped momentarily the bead jumps when boiling is resumed. The volume is 0.2 to 0.3 ml. The solution is transparent and pure blue.) (6) Cool one minute or longer. (7) Add 1 to 2 ml. water from a wash bottle. (8) Wash funnel inside and out with about 15 ml. distilled water. (9) Cool to 25° C. in a water bath. (10) Into a similar Pyrex tube put 2 ml. working standard solution, and into a third tube put 4 ml. of standard solution. To each add about 15 ml. distilled water and 0.5 ml. digestion mixture. To each of the three tubes: (11) add 1 ml. persulfate solution, (12) add 1 ml. potassium gluconate solution, (13) add distilled water to the 20-ml. mark, and (14) add 5 ml. Nessler's solution from a buret. (15) Stopper the tubes, mix at once, and let stand 10 minutes, to cool to room temperature. (16) Read in colorimeter, using a filter to admit light of 500 mμ wave length if photoelectric colorimeter is used. Read against the standard most nearly corresponding to the color of the unknown. If the latter is much darker than the standard, repeat, using a smaller volume of filtrate and correct the subsequent calculations accordingly. If the solution shows the slightest clouding it must be discarded and the test repeated.

Calculation, if the weaker standard solution is used, with a photoelectric colorimeter:

$$\text{N.P.N. in mg. per 100 ml.} = \frac{\text{Reading of unknown} \times 30}{\text{Reading of standard}}$$

With a visual colorimeter, if the unknown is set at 30, the reading of the standard gives directly the N.P.N. in mg. per 100 ml.

REAGENTS: DIGESTION MIXTURE. Dissolve 55 Gm. nitrogen-free sodium sulfate in 800 ml. distilled water. Add 500 ml. concentrated nitrogen-free sulfuric acid. Add 50 ml. 85 per cent phosphoric acid (syrup). Add 55 ml. 3 per cent copper sulfate. Cool. Dispense from an automatic buret.

PERSULFATE SOLUTION. Dissolve 2.5 Gm. nitrogen-free potassium persulfate in 100 ml. distilled water. Make up fresh weekly, and store in the icebox. Fill small dropping bottle for use each day, and discard excess.

POTASSIUM GLUCONATE SOLUTION. Dissolve 2.5 Gm. potassium gluconate in 100 ml. distilled water. Make fresh solution weekly and store in the icebox. Fill a buret daily, discarding the excess.

NESSLER'S SOLUTION. This solution is the same as that used in the Folin-Wu method (p. 766).

STANDARD NITROGEN SOLUTION. Dissolve 4.7160 Gm. dry ammonium sulfate (which contains 1 Gm. nitrogen) in 1000 ml. water.

WORKING STANDARD. Introduce 3 ml. of the above standard solution into a 100-ml. flask and dilute to the mark. These solutions keep indefinitely if tightly stoppered. Two ml. of the working standard contains 0.06 mg. nitrogen, which corresponds to the nitrogen present in 2 ml. of Folin-Wu filtrate of blood containing 30 mg. nonprotein nitrogen per 100 ml.

Urea Nitrogen (Gentzkow). (1) Pipet 2 ml. oxalated whole blood into a 125-ml. flask. (2) Add 13.5 ml. water. (3) Add 0.5 ml. urease solution. (4) Mix, cover with a beaker, and incubate at 37° C. for 30 minutes. (5) Add 2 ml. ⅔ N sulfuric acid. (6) Add 2 ml. 10 per cent sodium tungstate solution. (7) Shake well, and after a few minutes filter. (8) Pipet 5 ml. filtrate into a large Pyrex test tube graduated at 20 ml. (9) Into a second, similar tube pipet 2 ml. working ammonium sulfate solution, and into a third tube pipet 4 ml. standard solution. To each of the three tubes: (10) add 0.5 ml. N.P.N. digestion mixture, (11) add 1 ml. persulfate solution, (12) add 1 ml. potassium gluconate solution, and (13) add water to the 20-ml. mark. (14) To each tube add 5 ml. Nessler's solution and mix. (15) After 10 minutes read in the colorimeter, using the standard solution nearest to the unknown in color. If the color of the unknown is much darker than the standard, discard and repeat the procedure beginning with step 8, using a smaller volume of filtrate, and alter the calculation accordingly.

Calculation with a photoelectric colorimeter, if the more dilute working standard is used:

$$\text{Urea nitrogen in mg. per 100 ml.} = \frac{\text{Reading of unknown} \times 12}{\text{Reading of standard}}$$

With a visual colorimeter, if the unknown is set at 24, the reading of the standard divided by two gives the urea nitrogen in mg. per 100 ml. blood.

To express as urea, multiply this figure by 2.14.

SOLUTIONS: BUFFER PHOSPHATE MIXTURE. In a 100-ml. volumetric flask put 14 Gm. sodium pyrophosphate ($Na_4P_2O_7:10H_2O$) and 2 ml. 85 per cent phosphoric acid; dissolve in water, dilute to the mark, and mix. This ensures complete conversion of the urea.

UREASE. In a 250-ml. flask put 10 Gm. permutit; wash once with 2 per cent acetic acid and twice with water. Add 15 Gm. Jack bean meal and 100 ml. 16 per cent alcohol. Stopper and shake well for 30 minutes. Filter and store in the icebox. Prepare freshly every two weeks. When only occasional tests are made, it is advisable to cut heavy porous filter paper into strips 1 inch wide, saturate with the filtrate, dry at room temperature, and cut into 1.5-inch lengths. These keep active for many months. Reliable urease tablets can be purchased. The activity of such preparations should be checked occasionally.

Since many urease preparations contain nonprotein nitrogen, this filtrate cannot be used for determining nonprotein nitrogen unless the urease is tested and shown to be free from nonprotein nitrogen. Squibb's "double-strength" urease is free from nonprotein nitrogen and is recommended. The urease does not seem to interfere with the determination of the other constituents of the filtrate.

OTHER SOLUTIONS. The other solutions are identical with those used in nonprotein nitrogen determinations.

MODIFICATION OF UREA-NITROGEN DETERMINATION. If the Folin-Wu method of determining nonprotein nitrogen is used, it will be more convenient to alter the urea-nitrogen procedure as follows: Carry out steps (1) to (7) inclusive, as above. (8) Pipet 5 ml. filtrate into a large Pyrex tube graduated at 20 ml. (9) Into a 50-ml. volumetric flask pipet 3 ml. working standard solution containing 0.15 mg. nitrogen (p. 767). (10) To the unknown add 2 drops and to the standard add 4 drops gum ghatti solution. (11) Dilute the unknown to about 20 ml. and the standard to about 40 ml. with water. (12) To the unknown add 2.5 ml. Nessler's solution and to the standard 5 ml. (13) Dilute each to the mark, mix, and read in the colorimeter. The calculation is the same as above.

Significance of Nonprotein Nitrogen and Urea Nitrogen Findings. Normal individuals on an average diet show a nonprotein nitrogen of from 20 to 35 mg. per 100 ml. of blood, and a urea nitrogen from 10 to 20 mg. The quantity of these substances present is affected by several different factors: (1) the integrity or impairment of renal function, particularly as shown by the concentrating power and capacity to excrete urea; (2) the volume of urine excreted; (3) the occurrence and rate of destruction of body protein; (4) to some extent, the amount of protein in the diet.

In normal individuals a very low protein diet which is adequate in caloric value may lower the N.P.N. to 13 mg. %. A reduction of the N.P.N. to normal figures may sometimes be brought about in a similar manner in patients with a marked impairment of renal function, although this is detrimental to the patient's welfare. An abundant fluid intake with free diuresis has a potent effect in preventing accumulation of N.P.N. in conditions which tend to produce it.

Destruction of body protein occurs in a great variety of pathologic conditions, and, if marked in degree, may cause a substantial rise in N.P.N. if the urine volume is small.

When associated with marked oliguria, the N.P.N. may reach levels as high as those usually seen in extreme renal insufficiency. Among the more important conditions in which destruction of body protein may lead to an increase in N.P.N. without significant renal injury may be mentioned: starvation, especially with fluid restriction; poisoning with various drugs; sometimes after the administration of potassium iodide; surgical shock; acidosis; peritonitis; severe infections of all types; intestinal or pyloric obstruction; dehydration from any cause; intractable vomiting or diarrhea, as in cholera or dysentery; acute hepatic necrosis; and the terminal stage of Addison's disease. In acute hepatic necrosis the urea nitrogen has been reported to be relatively less elevated than the N.P.N., or in the terminal stage greatly reduced, the amino acids being greatly increased. In practically all other conditions the increase is mainly in the urea fraction.

In such *infections* as pneumonia a N.P.N. as high as 70 mg. % has been reported, without evidence of renal injury and with an essentially normal, concentrated urine. By increasing the fluid intake and securing diuresis, the figure may be brought to normal in two or three days, without any other notable change in the patient's condition. It is especially in dehydration associated with *obstruction to the gastrointestinal tract* and with vomiting of hydrochloric acid that excessively high figures have been observed. Peters and Van Slyke mention a case of pyloric obstruction with a N.P.N. of 292 mg. % and Volhard reported almost as high figures in severe diarrhea. If diuresis can be established by parenteral administration of salt solution, such figures can be speedily lowered. The persistence of a high N.P.N. after an operation for relief of peritonitis or intestinal obstruction (if renal function as determined by phenolsulfonephthalein excretion is good) is regarded as an unfavorable sign. The height of the N.P.N. is one indication of the devastating effect of dehydration in such conditions, although less significant than the fall in chlorides (and rise in carbon dioxide) which accompanies the rise in N.P.N. and which is probably its direct cause. High N.P.N. figures are rarely long maintained in such conditions, since the latter usually terminate quickly, either with death or recovery. (See section on blood chlorides and acid-base equilibrium.)

Chronic passive congestion, if uncomplicated, causes little or no rise in N.P.N., unless it is extreme. If it develops in the course of an infection, or in an individual with some degree of renal injury, it may precipitate a marked and rapid rise in N.P.N. There may be a sharp transient rise in N.P.N. during or immediately after a diuresis which has eliminated an edema (from any cause), probably because the water of the edema fluid can be excreted faster than the N.P.N. substances it contains, and the latter temporarily accumulate in the blood.

An elevation of the N.P.N. in nephritis occurs only with marked renal insufficiency. It is a late symptom and of no value in the early diagnosis of nephritis. Experimentally, a marked and sustained rise occurs only after about three-fourths of the kidney tissue has been destroyed. Focal lesions, therefore, must be very extensive before they will cause a rise in N.P.N. In the case of diffuse glomerular nephritis, also, about three-fourths of the renal function is lost before a marked rise in N.P.N. occurs. This rise is not a direct indication of the type of lesion in the kidneys, although high figures most often occur in the advanced stages of chronic glomerular nephritis, and contracted kidney due to arterial disease. In the terminal stages it may reach 100 to 300 mg. % or even higher. If one can exclude some transient remediable complication which may raise the N.P.N., a figure over 100 mg. % makes the immediate outlook grave. However, it is not the high N.P.N. in itself which directly causes uremia. It is important that such patients receive a diet adequate in caloric value and containing sufficient protein to prevent wastage of body protein, regardless of how high the N.P.N. may be. Excessive protein restriction is harmful. The nature of the supposed toxic substance which causes uremia is still a complete mystery. It is quite possible that some of the symptoms may be due to leakage of essential substances through the damaged kidneys.

No valid conclusions as to the significance of a high N.P.N. can be drawn without

considering all the other available information about the patient, including the nature of his diet, the volume of urine, and the presence of infection, circulatory failure, or other significant complications. Practically it is superfluous to determine both the N.P.N. and the urea nitrogen. Urea nitrogen must be determined if the urea clearance is to be estimated, and the N.P.N. if the plasma proteins are to be measured, or if the liver is damaged. Otherwise it is immaterial which procedure is chosen. The significance of the N.P.N. in nephritis is discussed further in connection with tests of renal function (p. 846).

Creatinine

Procedure. (1) Pipet 10 ml. Folin-Wu filtrate into a test tube. (2) Into a second tube pipet 5 ml. working standard solution, and (3) add 15 ml. distilled water. (4) To tube 1 add 5 ml. alkaline picrate solution, and (5) to tube 2 add 10 ml. (6) After 10 minutes read in the colorimeter.

As the intensity of the color in this reaction does not follow Beer's law closely, to avoid gross errors the color of the standard and of the unknown must approximately correspond. If a high reading is unexpectedly obtained, the test should be repeated, using a suitably smaller volume of filtrate. If this is impracticable, immediately add to the unknown a suitable measured amount of diluent consisting of one part alkaline picrate solution and two parts water, and read in the colorimeter. The calculations must be altered accordingly.

Calculation with photoelectric colorimeter:

$$\text{Creatinine in mg. per 100 ml. blood} = \frac{\text{Reading of unknown} \times 1.5}{\text{Reading of standard}}$$

With a visual colorimeter, if the unknown is set at 15, the reading of the standard divided by 10 gives the creatinine in mg. per 100 ml.

Reagents: Standard Solution—Stock. Dissolve 0.1 Gm. creatinine in 100 ml. of N/10 hydrochloric acid.

Working Standard Solution. Pipet 3 ml. stock solution into a 500-ml. volumetric flask, add 50 ml. N/10 hydrochloric acid, and dilute to the mark with water. Add a few drops of toluol as a preservative.

Alkaline Picrate Solution. To 25 ml. saturated picric acid solution add 5 ml. 10 per cent sodium hydroxide and mix.

Significance. It has been shown that the substance in blood which gives this color reaction is largely not creatinine, but this does not affect the practical significance of the reaction. The normal figures are from 1 to 2 mg. %. Creatinine is relatively little influenced by diet, or by extrarenal conditions which may increase the N.P.N. It is increased in cases of nephritis with marked impairment of renal function, rising about in parallel with the urea nitrogen. Figures over 5 mg. % in advanced chronic nephritis usually indicate impending uremia (see p. 846).

Uric Acid

Procedure (Folin). (1) Pipet 5 ml. blood filtrate prepared from serum into a test tube graduated at 25 ml. (2) Into a similar tube pipet 5 ml. working standard solution. (3) To each tube add from a buret 10 ml. urea-cyanide solution (poison) and mix. (4) To each add from a buret 4 ml. uric acid reagent, holding the tubes vertically. (5) After 20 minutes dilute to volume, mix, and read in colorimeter.

Calculation with a photoelectric colorimeter:

$$\text{Uric acid in mg. per 100 ml.} = \frac{\text{Reading of unknown} \times 4}{\text{Reading of standard}}$$

With a visual colorimeter, if the unknown is set at 20, the reading of the standard divided by 5 gives the uric acid in mg. per 100 ml.

Reagents: Urea-cyanide Solution. In a large (2-liter) beaker put 75 Gm. sodium cyanide (reagent), add 700 ml. distilled water, and stir until dissolved. Add 300 Gm. urea and stir, adding gradually 4 to 5 Gm. calcium oxide. Stir for 10 minutes, and let stand overnight. Filter, and add 2 Gm. lithium oxalate, shake occasionally for two hours, and again filter. This keeps several months in the icebox.

Lithium Oxalate. In a 3-liter beaker put 50 Gm. lithium carbonate and 85 Gm. crystallized oxalic acid. Pour on the mixture 1 liter of hot distilled water, and stir gently. After liberation of carbon dioxide ceases, filter. The filtrate may be evaporated to dryness and the residue powdered; or the lithium oxalate may be precipitated by adding 1 liter of alcohol, and the mixture filtered through a Buchner funnel.

Uric Acid Reagent. In a 500-ml. Florence flask put 100 Gm. sodium tungstate (molybdate free, Mallinckrodt Chemical Co.). To 150 ml. water add 32 ml. 85 per cent phosphoric acid. Pour this onto the sodium tungstate, shake, and boil for one hour under a reflux condenser. This may be improvised by putting in the mouth of the flask a 10-cm. funnel carrying a 200-ml. flask of cold water. Add enough bromine water to decolorize, boil off the excess bromine, cool, and dilute to 500 ml.

To test the reagent: In a test tube, put 5 ml. water, 4 ml. reagent, and 10 ml. urea-cyanide solution, and let stand 15 minutes. If a blue color develops, adjust reagent by adding 5 Gm. sodium tungstate, boil for 15 minutes, decolorize with bromine water if necessary, cool, and dilute to 500 ml.

Uric Acid Stock Standard Solution. Dissolve 0.6 Gm. lithium carbonate in 150 ml. hot water, filter, and heat to 60° C. Weigh just 1 Gm. uric acid on a watch glass, and transfer to a 1-liter volumetric flask using a small funnel, both the flask and funnel being warmed. Rinse in with the hot lithium carbonate solution, and immediately shake until dissolved (about five minutes). Cool under the tap, add 20 ml. 40 per cent formalin. Half fill the flask with water, and add a few drops of methyl orange. Add slowly from a pipet, shaking constantly, 25 ml. N sulfuric acid. The solution should turn pink while 2 to 3 ml. of acid are still in the pipet. Dilute to volume and mix. If stored in dark glass-stoppered bottles in the icebox, it is said to keep for several years.

Working Standard. Pipet 1 ml. of the stock standard solution into a 250-ml. volumetric flask and dilute to volume with water. Renew frequently. (The stability of the solution may be increased by adding 10 ml. ⅔ N sulfuric acid and 1 ml. formalin before bringing to volume.)

Significance. Normal blood contains from 2 to 4 mg. % of uric acid. The uric acid in the blood and tissue fluids is derived in part from the purins of the food (exogenous), and the balance (largely at least) from the disintegration of nucleic acid from the body tissues (endogenous). A *diet* high in purins has little effect on the blood uric acid in normal individuals, but increases it somewhat in gout and in nephritis. A diet high in purin-free protein, or in carbohydrate increases uric acid excretion, and lowers blood uric acid. On the other hand, a diet high in *fat* has the opposite effect and may raise blood uric acid to 10 or 12 mg. %. Starvation has a similar effect. In *gout* there is usually a moderate increase, from 4 to 10 mg. %. The figures are not always definitely abnormal, and may at times be reduced to some extent by dietary treatment or by the administration of salicylates or cinchophen. In *nephritis* there is often a moderate increase, even in cases with only slight impairment of renal function. Blood uric acid may rise to 10 or even 20 mg. % in uremia. However, there is no close parallelism between the height of the blood uric acid and the degree of renal insufficiency. High figures have been reported frequently in the toxemias of pregnancy, in leukemia, polycythemia, pneumonia, chronic passive congestion, and occasionally in a variety of other conditions. In general a normal blood uric acid is more valuable in excluding gout, than a high figure, in proving it.

Glucose

Tests for the quantitative determination of sugar in the blood are based on its property of reducing cupric salts in alkaline solution. There are other reducing substances present in the blood, particularly in the cells, which contribute to the reaction, and make the blood sugar figures erroneously high. In the Folin-Wu and other early methods the error ranged from 20 to 30 mg. %. The new methods of Benedict, and of Folin-Wu (which follows), reduce this to about 10 or 12 mg. %, and consequently give blood sugar readings about *20 mg. % lower* than the older methods, on which published statistics and discussions in the literature are based. This fact must be remembered in interpreting the results of the newer and more accurate procedures.

Determinations may be made either on venous blood, as is the usual custom in this country, or on "capillary" (finger-tip) blood, as has been customary in Europe. Equally precise determinations can be made with blood from either source, but except in the fasting state they do not give identical results, venous blood containing less sugar than capillary blood, which is practically arterial blood. This is discussed in detail in connection with glucose tolerance tests.

Folin-Wu Method (Applicable to Venous Blood). (1) In a Folin sugar tube put 2 ml. blood filtrate. (2) In a second tube put 2 ml. single glucose standard solution, and (3) in a third tube put 2 ml. double standard. (4) To each add 2 ml. freshly mixed copper tartrate solution. (5) Heat in boiling water bath for eight minutes. (6) Cool in water. (7) Add 4 ml. acid molybdate reagent. (8) After one minute dilute to volume with *diluted reagent*. (9) Mix and compare in colorimeter.

Calculation with a photoelectric colorimeter, if the single standard was used:

$$\text{Sugar in mg. per 100 ml.} = \frac{\text{Reading of unknown} \times 100}{\text{Reading of standard}}$$

With a visual colorimeter, if the unknown is set at 20, the reading of the standard multiplied by 5 gives the sugar in mg. per 100 ml.

REAGENTS: STANDARD SOLUTION OF GLUCOSE (STOCK). In a volumetric flask dissolve 1 Gm. anhydrous glucose (Bureau of Standards) in 100 ml. saturated benzoic acid solution. 1 ml. = 10 mg. It keeps for years.

INTERMEDIATE STANDARD. Dilute 100 ml. stock solution to 500 ml. with benzoic acid solution. This also keeps indefinitely. 1 ml. = 2 mg.

DOUBLE WORKING STANDARD. Freshly dilute 10 ml. of the preceding to 100 ml. with half saturated benzoic acid solution. 1 ml. = 0.2 mg.

SINGLE WORKING STANDARD. Dilute 5 ml. of the intermediate standard solution to 100 ml., similarly. 1 ml. = 0.1 mg.

COPPER SULFATE SOLUTION. Dissolve 25 Gm. crystallized copper sulfate in some water in a 500-ml. volumetric flask, add 5 drops concentrated sulfuric acid, dilute to volume.

ALKALINE TARTRATE SOLUTION. In a 1-liter volumetric flask put 35 Gm. anhydrous sodium carbonate, add 200 ml. water, and shake until dissolved. Add 13 Gm. sodium tartrate (Merck's highest purity) and 11 Gm. sodium bicarbonate. Add about 600 ml. more water, shake until clear, dilute to volume, and mix.

ALKALINE COPPER TARTRATE SOLUTION. Prepare daily by adding 9 volumes of the alkaline tartrate solution to 1 volume of the copper sulfate solution.

ACID MOLYBDATE REAGENT. (1) In a 1-liter volumetric flask put 300 Gm. sodium molybdate, add about 800 ml. water, and shake until dissolved. Dilute to the mark, mix, transfer to a bottle, and add 0.3 ml. liquid bromine, shake, and let stand. (2) In a large flask put 500 ml. of the clear supernatant fluid, and add, while stirring, 225 ml. 85 per cent phosphoric acid. Then add 150 ml. cool 25 per cent (by volume) sulfuric acid, and let stand overnight. Pass an air current through the solution to remove the

bromine. Add 75 ml. 99 per cent acetic acid. Mix, and dilute to 1 liter in a volumetric flask. This keeps indefinitely.

DILUENT. Add 1 volume of this reagent to 4 volumes water.

Folin's Ferricyanide Micro-method. (1) In a centrifuge tube put 4 ml. sulfate-tungstate solution. (2) Fill to the mark a pipet accurately graduated to *contain* 0.1 ml. with blood from the finger tip (or vein), wipe off any blood that may adhere to the outside of the pipet, and discharge the blood into the tungstate solution. Rinse out the pipet with solution in the tube. Mix. (3) After 15 minutes or longer add 1 ml. sulfuric acid-sulfate solution, mix, and centrifuge for five minutes. (4) Put 2 ml. of the clear solution and 2 ml. water in a test tube graduated at 25 ml. In a similar tube put 4 ml. standard glucose solution. (5) To each add 1 ml. 0.4 per cent potassium ferricyanide solution (2 ml. if a high blood sugar is expected), and (6) 1 ml. cyanide-carbonate solution. (7) Heat in boiling water bath eight minutes; cool in running water for two minutes. (8) Add 5 ml. ferric iron solution and mix. (9) After two minutes dilute to the mark (adding 3 drops of alcohol to get rid of foam as the mark is approached, if necessary) and mix. (10) Put standard solution in both colorimeter cups, set both at 20, insert yellow light filter, and adjust light until they are exactly alike. (11) Read unknown in the colorimeter.

Calculation:

$$\frac{\text{Standard}}{\text{Unknown}} \times 100 = \text{mg. \% glucose.} \quad \left(\text{For great precision:} \frac{101 \text{ Stand.}}{\text{Unkn.}}\right)$$

This method gives proportionately accurate readings with the unknown solution within the range of 5 mm. to 40 mm. provided the light and color filter are properly adjusted. It gives slightly higher readings than the preceding method. Without the light filter it is necessary to prepare numerous standard solution tubes of varying concentration, and use one that closely approximates the color of the unknown solution.

REAGENTS: SULFATE TUNGSTATE SOLUTION. In a 500-ml. volumetric flask, put 10 Gm. anhydrous sodium sulfate, and 15 ml. 10 per cent sodium tungstate solution. Dissolve in water, fill to volume and mix.

SULFURIC ACID. In a 100-ml. volumetric flask put 12 ml. ⅔ N sulfuric acid, and 2 Gm. anhydrous sodium sulfate, dissolve, fill to volume with water, and mix.

POTASSIUM FERRICYANIDE SOLUTION. Dissolve 2 Gm. *pure* salt in water, and dilute to 500 ml. Keep in the dark in brown bottles.

CYANIDE-CARBONATE SOLUTION. Put 8 Gm. anhydrous sodium carbonate in a 500-ml. flask, add 50 ml. water, shake till dissolved. Add 150 ml. freshly prepared 1 per cent sodium cyanide solution. Fill to the mark, mix, filter if necessary.

FERRIC IRON SOLUTION. Fill a 1-liter graduate with water. Put 20 Gm. gum ghatti in a tea ball, and suspend in the top of this water for 18 hours. Filter through a double layer of clean towel. Add to the extract a solution containing 5 Gm. anhydrous ferric sulfate dissolved in 75 ml. 85 per cent phosphoric acid and 100 ml. water. To remove reducing substances in the gum ghatti, add a little at a time about 15 ml. 1 per cent potassium permanganate solution, until the pinkish tint persists for some minutes. Turbidity if present will clear if kept at 37° C. for a few days.

STANDARD GLUCOSE SOLUTION. This contains 0.01 mg. per 1 ml. Dilute to 200 ml., 1 ml. of the intermediate standard (B) prepared for the previous method, using 25 ml. of 0.2 per cent benzoic acid and water to the mark. Keep under toluol. Renew frequently.

YELLOW LIGHT FILTER. A suitable glass filter can be purchased (Spencer Lens Co., Buffalo, N. Y.).

Significance. The normal fasting blood sugar (either in venous or capillary blood) ranges from 70 to 120 mg. % by the older methods, and from 60 to 100

mg. % by the methods above given. In elderly individuals the normal maximum figure is from 10 to 20 mg. higher. It may be reduced by starvation, particularly in children, even to 40 mg. %. Lower figures are also seen in normal individuals four to six hours after a meal. A transient rise in blood sugar may follow exposure to cold or extreme heat, painful stimuli, emotional disturbances of any sort, asphyxia, and the administration of various drugs, such as general anesthetics, morphia, amytal, caffeine, quinine, *epinephrin,* and *ephedrin.*

A persistent *increase in blood sugar* is most often seen in diabetes, the fasting sugar ranging in untreated individuals from 100 to 300 mg. % in the milder cases, and up to 1200 mg. % in the most severe. A pathologic increase may also occur in hyperthyroidism and hyperpituitarism, some adrenal tumors, in some acute infections, particularly pyodermias, in chronic diseases of the liver and bile ducts, in some malignant neoplasms, in various intracranial lesions, and in some cases of essential hypertension with good renal function and of chronic nephritis with hypertension, especially with nitrogen retention.

Pathologically low figures, from 60 to 30 mg. %, have been reported in cases of neoplasms of the pancreas involving the islands of Langerhans, accompanied by excessive insulin production, and characterized clinically by severe hypoglycemic reactions. A similar milder clinical disturbance with a lesser reduction in blood sugar is not rare in individuals without gross lesions of the pancreas, and has been attributed to functional overactivity of the islands. The blood sugar may be low in cases of hypopituitarism and of Addison's disease.

Blood sugar estimations are of greatest practical value in the recognition of diabetes and in controlling its treatment. Before the diagnosis of diabetes can be established other causes of hyperglycemia must be excluded.

Carbohydrate Metabolism

The carbohydrates of the food are absorbed chiefly as hexoses: glucose, fructose, and galactose. Small amounts of other sugars are also absorbed. Except for maltose, the disaccharides (sucrose, lactose, etc.) cannot be utilized by the tissues, and if absorbed they are totally excreted in the urine.

After absorption these sugars are carried by the portal circulation to the liver where a large proportion is abstracted and converted into glycogen. A high blood sugar seems to be the immediate stimulus for this conversion.

Maintenance of a normal concentration of glucose in the blood and tissues depends upon the breakdown of glycogen into glucose and its orderly release by the liver. A low blood sugar supplies the stimulus for this glycogenolysis.

These processes are controlled by the interaction of various hormones of which insulin and epinephrin have been most carefully studied and are probably of major importance. The anterior pituitary, the thyroid, and probably the adrenal cortex participate.

The major functions of *insulin* are: (1) It inhibits the conversion of glycogen into glucose by the liver. (2) It stimulates the formation of glycogen in the liver. (3) It stimulates the conversion of glucose into muscle glycogen. (4) It increases

the oxidation of glucose or its decomposition products in the tissues. As a result of the last two functions, a hypoglycemia tends to develop, and this may lead to a secondary glycogenolysis and depletion of liver glycogen.

In the *diabetic* these functions of insulin in large measure are lost. There is a gross impairment of the capacity to form and store glycogen and to oxidize glucose. Fats also are incompletely oxidized, and lipemia, ketosis, and acidosis result. It is not yet certain which of these disturbances is primary. It has been commonly assumed that the primary defect in diabetes is the loss of the power to oxidize glucose, and that the other disturbances are secondary to this. This view is based in part on the low respiratory quotient (R.Q.) which is believed to indicate that only fat is being oxidized, and on the fact that the R.Q. is elevated by insulin. However, the R.Q. may be influenced by other factors than the composition of the food mixture which is being oxidized, which are difficult to control experimentally. There is considerable experimental evidence that the totally diabetic animal retains to some extent the capacity to oxidize glucose and to form glycogen. Such facts have led some to believe that the primary disturbance is unrestricted gluconeogenesis, an overproduction of glucose, and that deficient oxidizing power is a secondary result.

There is also controversy as to whether all cases of diabetes in human beings are due to primary disease of the pancreas. Significant lesions of this organ are not uniformly found at autopsy. Some cases of diabetes are unusually refractory to the therapeutic action of insulin. Progressive diabetes can be produced experimentally in animals by repeated injections of anterior pituitary extract. Further study may show that some cases in human beings are due to overactivity of the anterior pituitary. It seems improbable that true diabetes results from a primary disturbance of the thyroid, the adrenal, or the liver, although hyperglycemia and glycosuria occur in some diseases of these organs.

Glucose Tolerance Test. The glucose tolerance test was devised as a clinical test of the integrity of the mechanism controlling the assimilation of glucose. The normal assimilation limit of glucose has been determined precisely in animals by continuous intravenous injections of glucose. If the rate of injection is 0.8 Gm. per kilogram per hour or less, no glucose is excreted in the urine. If the rate of injection is from 0.9 to 2.0 Gm. per kilogram per hour, some glucose is excreted, the amount increasing as the rate of injection rises but not exceeding one-tenth of the amount injected. If more than 2 Gm. per kilogram per hour is injected, a large proportion of the excess over 2 Gm. is excreted. Since the maximum rate of absorption from the intestine never exceeds 1.8 Gm. per kilogram per hour, no notable loss of glucose in the urine is to be anticipated after its administration by mouth in any amount to a normal individual.

When a normal individual takes a substantial amount of glucose (50 Gm. or more) by mouth the blood sugar quickly rises. As it rises, it stimulates the process of conversion of the excess of glucose into glycogen and its storage in the liver and muscles. This process goes on with increasing rapidity, and soon equals and exceeds the rate of absorption from the intestine. As a result the blood sugar begins to fall sharply and

abruptly (in 20 to 40 minutes), as a rule before the rising renal threshold for glucose has been exceeded (see p. 833). The storage process continues until the blood sugar (after the third or fourth hour) has fallen well below the fasting level.

To map out the course of the blood sugar precisely and particularly to determine the peak of the curve (which is maintained only for a few minutes) and the threshold level, if sugar is excreted, it is necessary to obtain blood (and urine) specimens at frequent intervals, at least every 10 minutes. For these purposes finger-tip blood is essential, because during its circulation through the muscles of the arm the blood loses a large and variable amount of glucose. The venous blood sugar at the peak (140 to 160 mg.) is from 30 to 70 mg. lower than the capillary blood sugar (170 to 220 mg.). As the blood sugar falls, this difference diminishes, but the venous blood sugar remains the lower until the fasting level is regained several hours later. It is manifestly the capillary blood sugar level which determines excretion of sugar by the kidney, and which is the more significant figure.

In normal individuals increasing the amount of glucose administered above 50 Gm. (to 100 or even 200 Gm.) does not, as one might expect, increase the height of the peak of the curve. Increasing the amount of glucose administered does not accelerate the rate of absorption. The storage mechanism, once in operation, can keep pace with the maximum rate of absorption. However, such large amounts prolong the period of absorption and delay the fall in blood sugar to the fasting level.

In diabetes the response is entirely different. The mechanism for storage of carbohydrate (as glycogen) is disturbed, as well as that for its oxidation. It is removed from the blood very slowly, and as a result the blood sugar rises rapidly to a much higher level, well above the rising threshold; the peak is flatter and more protracted; and the return to normal is much delayed, so that the blood sugar is still high three and four hours after the glucose administration. In diabetes the height of the peak does vary with the amount of glucose given, and there is less difference between the venous and capillary blood sugar. In mild cases every transition may be met with from a normal response to an outspoken diabetic curve.

The *diabetic response,* then, differs from the normal in two major respects. The peak is higher and the return to normal is much delayed. As a routine clinical procedure it is manifestly impracticable to make a sufficient number of observations to determine the peak precisely, and chief reliance must be placed on the delay in the return to normal. This varies materially with the dose of glucose administered. The usual custom has been to give 100 Gm. of glucose, or 1.75 Gm. per kilogram; but this quantity may well be somewhat injurious to an outspoken diabetic, and in normal cases it tends to obscure rather than emphasize the differences between a normal and a pathologic response. The optimum dosage is lower, preferably 1.25 Gm. per kilogram. The smaller quantities are also less likely to cause nausea. The only disadvantage is that the current standards of normal are based on tests with the larger quantities, and the criteria for tests with smaller quantities must be somewhat more strict. After 1.25 Gm. per kilogram the glucose in capillary blood returns to normal after two hours (average) to a maximum of two and one-half hours or, in elderly individuals, three hours. The venous blood sugar reaches normal 30 to 60 minutes sooner. Increasing the dose to 1.75 Gm. per kilogram prolongs the time about one hour. For determining the duration of the curve examinations of venous blood are satisfactory.

Procedure. A glucose tolerance test should be carried out whenever diabetes is suspected. It is usually inadvisable in frank cases. The test should be carried out in the morning, after a fast of 12 to 15 hours. The patient must have been on an unrestricted diet for several days previously, otherwise a normal individual may yield an abnormal curve. The patient should rest quietly during the test, and emotional disturbance must be avoided. (1) The patient's bladder is emptied and the urine saved, and blood is drawn for sugar determination, either from the vein or, preferably, from the finger if the

peak or threshold is to be determined. (2) The patient at once drinks a solution containing 1.25 Gm. glucose per kilogram of body weight, dissolved in about 250 ml. water. This may be flavored with a little lemon juice. (3) Collect urine and blood at 30 minutes, one hour, two hours, and three hours after the patient has taken the glucose (the two-hour specimen is the most important). If hypoglycemia is suspected, obtain specimens after four hours and five hours also. (4) Make quantitative determinations of glucose on all specimens. The patient may be allowed to have water as desired, but nothing else until the test is completed.

Interpretation. A normal individual shows a fasting blood sugar usually under 100 mg. %. The maximum figure observed (usually at the one-half-hour interval) does not exceed 180 mg. % in venous blood (220 mg. % in capillary blood) and may be very much less, since the actual peak is only occasionally observed when specimens are taken so infrequently. The figure drops substantially to the fasting level after about two hours in venous blood, and two to two and one-half hours in capillary blood, and well below this after three hours (often to 60 mg. % in venous blood). The amount of sugar excreted in the urine is of minor importance.

A normal curve practically excludes diabetes. No stress can be laid on slight deviations from the normal. The test is disturbed by variations in the rate of absorption of glucose. Particularly if it causes nausea, gastric motility may be altered so as to delay absorption and give a more protracted curve with a lower peak. A prolonged curve indicating impaired tolerance may be found in other conditions in which a high fasting blood sugar occurs, particularly in overactivity of the thyroid, pituitary, and adrenal, and in some cases of obesity and of hypertension and nephritis. In hyperthyroidism it is common to observe a high peak, with relatively little delay in the fall to normal.

In cases of *hyperinsulinism* the peak of the curve is lower than normal and the blood sugar falls to pathologically low figures (40 to 60 mg.) at the fourth, fifth, or sixth hour after administration of glucose. In *sprue* and *idiopathic steatorrhoea* (but not in pernicious anemia or pellagra) the curve is typically flat with a low peak.

Two-dose Tolerance Test (Exton and Rose). This test is preferred by some. In principle it depends upon the fact that a preliminary administration of carbohydrate accelerates the utilization of carbohydrate subsequently administered in a normal individual, but has the reverse effect in diabetes. (1) The patient should be prepared as for an ordinary tolerance test. (2) Dissolve 100 Gm. glucose in about 600 ml. water flavored with lemon juice, and divide the solution into two equal portions. (3) Obtain fasting specimens of blood and urine. (4) Administer one portion of the glucose (50 Gm.). (5) Just 30 minutes later obtain specimens of blood and urine, and administer the second portion of the glucose. (6) After 30 minutes again obtain specimens of blood and urine. (This completes the test unless hypoglycemia is suspected, in which case obtain four additional specimens at one-hour intervals.) Make quantitative determinations of glucose for all specimens.

Interpretation. In a normal individual the fasting blood sugar is within the normal limits already discussed; it rises not more than 75 mg. % after 30 minutes; and in the 60-minute specimen it is not more than 5 mg. % higher than in the 30-minute specimen and may be lower. Glycosuria is usually absent, but may occur in persons with a "low renal threshold." In diabetes, (1) the fasting blood sugar is usually more or less elevated (above 120 mg.), (2) it rises more than 75 mg. % after 30 minutes (according to Gould et al., more than 50 mg. %), and (3) there is a further rise of at least 10 mg. %

(according to Gould et al., at least 30 mg. %) in the final specimen. There is usually a substantial amount of glucose in the urine. If any two of these three criteria are abnormal, the test indicates the presence of diabetes. The precautions noted for the preceding method must be observed. It is claimed that this method gives fewer misleading positive reactions.

Renal Glycosuria ("Renal Diabetes"). This is a condition characterized by the excretion of glucose in the urine of individuals who show a normal (or low) blood sugar, and no disturbance in the capacity to oxidize and utilize carbohydrate (low renal threshold). The glycosuria is independent of the diet, and may occur during a fast. It may be transient or continuous. It is usually slight or moderate, but it may be marked. Glucose tolerance tests show normal or shortened curves. The condition is a constitutional anomaly which does not affect the health, shorten life, or predispose to diabetes. Carbohydrates should not be restricted. In its milder forms it is fairly common. Precise differentiation from diabetes is most important, and may require a considerable period of observation.

Chlorides

Determinations of chloride in whole blood are of less value than in serum or plasma because of the fact that the concentration of chloride in the cells is only about half that in the plasma, and differences in chloride content of whole blood due to differences in the volume of the cells associated with anemia or polycythemia overshadow those commonly met with in disease. However, chloride passes from the cells to the plasma when blood is exposed to the air, because of the escape of carbon dioxide from the blood. To avoid this error it is, therefore, preferable to collect the blood under oil and to remove the serum as promptly as possible without exposure to the air, and this is essential for research accuracy.

Procedure (Van Slyke and Sendroy). (1) Put in a large Pyrex test tube 1 ml. plasma or serum (or whole blood) which has been collected under oil and not exposed to the air until after separation from the cells. (2) Add slowly with constant stirring 3 ml. of the silver nitrate-nitric acid reagent. (3) Cover mouth of the tube with a watch glass, and put in a steam bath or boiling-water bath until the fluid over the silver chloride precipitate is clear and light yellow in color. (Requires one to two hours; if necessary decolorization can be completed by adding, a drop at a time, a few drops of saturated potassium permanganate solution.) (4) Cool to room temperature or lower. (5) Add 6 ml. 5 per cent ferric alum solution, or 6 ml. water and 0.3 Gm. powdered ferric alum. (6) Titrate excess of silver nitrate with 0.02 N sulfocyanate solution until a pink color is obtained that persists 15 seconds. The addition of 2 ml. nitrobenzene or chloroform before titration gives a sharper end point.

Calculation:

$$71 \times (7.54 - \text{number of ml. of sulfocyanate solution used}) = \text{mg. Cl per 100 ml. plasma.}$$

(To express as NaCl, substitute 117 for 71. To express as mM, substitute 20.)

Silver Nitrate-nitric Acid Reagent. Dissolve 8.495 Gm. fused silver nitrate in a minimum amount of water, and dilute to 1 liter with concentrated nitric acid. (It keeps indefinitely.)

0.02 N Sulfocyanate Solution. Dissolve 1.5 Gm. ammonium sulfocyanate in about 900 ml. water. Check the strength of the solution by titrating against 3 ml. of the silver nitrate-nitric acid solution and adjust so that just 7.54 ml. are required. This solution changes on standing and must be rechecked frequently.

Whitehorn's Method. Determination of the chlorides in *whole blood* can be made

from the Folin-Wu tungstic acid filtrate. (1) To 10 ml. filtrate add 5 ml. silver nitrate solution (containing 4.791 Gm. per liter, 1 ml. = 1 mg. Cl), mix, and add 5 ml. concentrated nitric acid. After 10 minutes add 0.3 Gm. powdered ferric alum, and titrate with standard NH_4CNS solution. This is prepared by dissolving 2.3 Gm. ammonium sulfocyanate in a liter of water, and adjusting by titration and dilution so that 5.04 ml. corresponds exactly to 5.0 ml. of the silver nitrate solution. The end point is a salmon-red tint that persists for 15 seconds in spite of stirring.

Calculation:

(5 — No. of ml. of sulfocyanate solution used) × 100 = mg. Cl per 100 ml. of whole blood.

Significance. The *normal chloride content* of whole blood is 273 to 303 mg. per 100 ml. and of plasma 352 to 383 mg. %, corresponding to 560 to 630 mg. % of sodium chloride, or 99 to 108 millimoles (mM) per liter. The extremes of normal are from 95 to 110 mM, and fluctuations in disease usually do not exceed these limits materially. Hence great precision in technic is required to get determinations of any value.

One of the major *functions of sodium chloride* is to maintain the normal osmotic pressure in the body fluids. Some unknown mechanism in which the kidney must play the major role keeps the electrolyte content of the plasma nearly constant. Urine excretion of chloride practically stops when the plasma chloride falls below 96 mM. If chloride is ingested faster than it can be excreted, it does not accumulate in the plasma but passes (as sodium chloride and water) into the intercellular tissue spaces and produces edema. In chronic passive congestion, "nephrosis," and active chronic nephritis with edema this tendency is exaggerated, because the mechanism for the excretion of water and salt is impaired in these conditions.

Transient rises in plasma chloride, usually trivial in degree, have been described in a number of conditions, but are of little or no practical significance.

A significant reduction in chloride may occur in acute infections, chiefly pneumonia, in which it may fall to 80 mM.

A *marked reduction of chloride* may occur in severe diabetic ketosis, in which plasma chloride is replaced by organic acids and excreted in the urine. It also follows excessive loss of chloride through other channels than the kidney, as after profuse sweating resulting from heavy labor at high temperatures (heat exhaustion, stoker's cramps); or persistent diarrhea, as in cholera; in cases with duodenal fistula, and in extensive burns. Maximum depletion, even to half the normal figures, follows intractable vomiting, particularly when associated with pyloric or high intestinal obstruction, but also in toxemic vomiting of pregnancy, mercuric chloride poisoning, etc.

If hydrochloric acid is vomited, an alkalosis from loss of acid is also produced, with a high bicarbonate content in the plasma. If achlorhydria is present, there is no alkalosis, but there is an equal loss of plasma electrolytes, and lowering of both sodium and chloride. Although the depletion of base (chiefly sodium) is probably of primary importance, determinations of sodium are time-consuming and impracticable as a routine procedure. Peters has pointed out that the sum of the serum chloride and serum bicarbonate expressed in mM closely approximates

the total sodium of the serum, and (usually) is practically a measure of the blood electrolytes.

In these conditions parenteral injections of physiological or even hypertonic salt solution relieve symptoms and prolong life. The giving of water is harmful, since the body is unable to retain water without sufficient salt to make it isotonic with the body fluids. Neither will salt be retained or absorbed unless adequate water is also given.

In *essential hypertension* and in *chronic nephritis* without edema there is no change in plasma chloride or in chloride excretion except when renal insufficiency has become marked. Then the kidney loses its power to control chloride excretion. It is unable to concentrate sodium chloride normally in the presence of an excess, or to hold it back when plasma chloride is low. Hence either high or low figures may be met with, depending largely upon the sodium chloride intake. In uremia, plasma chloride is more often reduced, sometimes markedly so.

Plasma chloride determinations are of especial value in detecting and measuring the more marked degrees of chloride depletion in the conditions which have been enumerated, and in guiding their treatment with sodium chloride injections.

Calcium and Phosphorus

Calcium. Determinations are made on the *serum*. There is practically no calcium in the red cells.

Clark-Collip Modification of Kramer-Tisdall Method. (1) In a 15-ml. graduated centrifuge tube with a narrow tip (having an outside diameter of 6 to 7 mm. at the 0.1-ml. mark) put 2 ml. *clear* serum. (2) Add 2 ml. water and 1 ml. 4 per cent ammonium oxalate solution. (3) Mix thoroughly, and let stand two hours or longer. (4) Mix, and centrifuge at high speed for 10 minutes. (5) Decant the supernatant fluid carefully, taking care not to agitate the precipitate, put the inverted tube on a pad of filter paper, and drain for five minutes. (6) Wipe the mouth of the tube dry, wash down the walls of the tube with 3 ml. dilute ammonia solution, and resuspend the sediment. (7) Centrifuge and drain as described. If carefully done, the slight loss of sediment is compensated for by the trace of ammonium oxalate remaining in the precipitate. (8) Add 2 ml. normal sulfuric acid so as to break up and dissolve the precipitate, and heat in a boiling-water bath for a minute. (9) Keep the solution at 70° C. or higher, and titrate with a 0.01 N solution of potassium permanganate using an accurate 5-ml. microburet until a definite pink color lasting one minute is obtained. (10) Check the strength of the permanganate solution by titration with freshly prepared 0.01 N solution of sodium oxalate acidified with a few drops of strong sulfuric acid, and correct the reading. If, e.g., 10 ml. sodium oxalate solution (0.01 N) requires 11 ml. 0.01 N permanganate solution to give a pink color, the reading observed under (9) should be multiplied by $^{10}/_{11}$.

Calculation:

$$1 \text{ ml. of } 0.01 \text{ N } KMnO_4 \text{ solution} = 0.2 \text{ mg. calcium.}$$

$$\text{Buret reading} \times \left(0.2 \times \frac{100}{2} \right) = \text{mg. calcium per 100 ml. serum.}$$

Reagents: *Ammonium Oxalate Solution*. Dissolve 4 Gm. in 100 ml. water.

Ammonia Solution. Dilute 2 ml. of strong ammonia solution to 1 liter with water.

Normal Solution of Sulfuric Acid. Dilute 28 ml. of concentrated sulfuric acid to 1 liter with water.

N/10 Potassium Permanganate Solution (*Halverson and Bergeim*). Dissolve 4 Gm. pure potassium permanganate in 1 liter distilled water, in a Florence flask. Put funnel in the mouth of the flask, cover with a watch glass, heat barely to boiling, and digest for several hours. Cool, let stand overnight, and filter through glass wool, or through ignited asbestos in a Buchner funnel, by suction. Store in brown glass-stoppered bottles in a dark place. After a preliminary period of relatively rapid weakening this solution keeps almost unchanged for many months. Its strength must be checked by titration with N/10 sodium oxalate or oxalic acid solution and adjusted. (Theoretically it should contain 3.158 Gm. per liter.)

N/100 Potassium Permanganate Solution. Dilute 10 ml. of the N/10 solution to 100 ml. with distilled water. This solution weakens rapidly, and its strength must be checked daily. (It may not be filtered through paper. If turbid, centrifuge.)

N/10 Sodium Oxalate Solution. Dissolve 6.7 Gm. of the Sørensen salt in 1 liter water containing 5 ml. concentrated sulfuric acid. This keeps indefinitely.

N/100 Sodium Oxalate Solution. Dilute 10 ml. of the N/10 sodium oxalate solution to 100 ml. with water. This should be freshly prepared each week. N/100 oxalic acid solution may be used.

The *normal figures* are 9 to 11.5 mg. % for adults, and 10 to 11.5 mg. % for children.

SIGNIFICANCE. Calcium occurs in serum in two forms. One part (3 to 5 mg. %), which appears to be physiologically inactive, is kept in solution by a combination with plasma protein, and is diminished in any condition in which plasma protein is diminished. The balance, which is the physiologically active calcium, behaves as if it existed in plasma as $Ca_3(PO_4)_2$ in saturated solution in contact with undissolved $Ca_3(PO_4)_2$ (in the bones). Anything which causes a change in the amount of either calcium or inorganic phosphorus in the plasma causes a reciprocal change in the other, by abstracting it from, or depositing it in the bones, which act as a storehouse for these elements. Thus, administration of soluble calcium salts ($CaCl_2$) tends to raise blood calcium, and indirectly lower blood phosphorus, whereas administration of soluble acid phosphates tends to raise blood phosphorus, and lower calcium. To interpret the significance of changes in blood calcium, it is therefore necessary to know the amount of both total plasma protein and inorganic phosphorus present. These changes are discussed more fully in conjunction with phosphorus in the following pages.

Inorganic Phosphorus (Fiske and Subbarow). Pipet 2 ml. clear serum into a small flask, and add slowly 8 ml. 10 per cent trichloracetic acid. (2) Mix, and filter through Whatman acid-washed filter paper No. 30. (3) Pipet 5 ml. filtrate into a 10-ml. volumetric flask. (4) Pipet 5 ml. standard phosphorus solution into a 100-ml. volumetric flask and add about 65 ml. water. (5) To the filtrate add 1 ml. molybdate solution No. 2, and to the standard add 10 ml. molybdate solution No. 1, and mix. (6) To the filtrate add 0.4 ml. aminonaphtholsulfonic acid reagent, and to the standard, add 4 ml. (7) Mix, dilute both to volume, and mix. (8) After five minutes read in colorimeter.

Calculation, if photoelectric colorimeter is used:

$$\text{mg. \% P} = \frac{4 \times \text{Reading of unknown}}{\text{Reading of standard}}$$

With a visual colorimeter, if the unknown is set at 20, the reading of the standard divided by 5 gives the phosphorus in mg. %.

REAGENTS: TRICHLORACETIC ACID SOLUTION. A 10 per cent solution of trichloracetic acid is used.

SULFURIC ACID SOLUTION. To 650 ml. water add 225 ml. concentrated sulfuric acid. This gives a 10 N solution.

MOLYBDATE SOLUTION NO. 1. In a 1-liter volumetric flask dissolve 25 Gm. ammonium molybdate in about 200 ml. water; add 500 ml. 10 N sulfuric acid, cool, dilute to the mark, and mix.

MOLYBDATE SOLUTION NO. 2. Prepare as above, using 300 ml. 10 N sulfuric acid.

AMINONAPHTHOLSULFONIC ACID REAGENT. Dissolve 0.5 Gm. aminonaphtholsulfonic acid in 195 ml. 15 per cent sodium bisulfite solution in a 250-ml. glass-stoppered bottle; add 5 ml. 20 per cent sodium sulfite solution, and shake until dissolved. If necessary add 1 ml. more of the sodium sulfite solution. This must be freshly prepared every two weeks.

STANDARD PHOSPHORUS SOLUTION. In a 1-liter volumetric flask, put 0.3509 Gm. monopotassium phosphate and dissolve in some water. Add 10 ml. 10 N sulfuric acid. Dilute to the mark and mix. This keeps well.

SIGNIFICANCE. The *normal figures for inorganic phosphorus in serum* or plasma in adults range from 2.5 to 5.0, usually about 3.5 mg. per 100 ml., and in children from 4 to 7, usually about 5 mg. %. The phosphorus in organic combination in the blood, particularly in the red cells, is far greater than the inorganic phosphorus, but thus far no information of practical clinical value has been obtained from its estimation. This method, if applied to whole blood, includes a large amount of acid-soluble organic phosphorus from the cells, and gives figures which are far higher than for serum and are at present meaningless. The figures which follow refer to inorganic serum phosphorus.

Significance of Blood Calcium and Inorganic Phosphorus. The amount of calcium phosphate dissolved in plasma is about three times the quantity which will dissolve in an aqueous solution having the same salt concentration and pH as the plasma. Its greater solubility in plasma is attributed to the action of the parathyroid hormone. Most conditions showing abnormalities in blood calcium which are not merely secondary to reduced plasma proteins or to primary changes in phosphorus are attributable to disturbances of parathyroid function. Injections of parathormone, or spontaneous *hypersecretion of the parathyroids* met with in adenomas of these glands, cause a marked rise in blood calcium, to 14 to 18 mg. %. This is associated with increased excretion in the urine and with extensive decalcification of the bones, which may lead to diffuse osteoporosis, multiple localized rarefied cystic areas, and spontaneous fractures. There is often pathologic deposition of calcium in other tissues and formation of renal calculi. There is muscular weakness and hypotonia. The phosphorus is usually low, but after large doses of parathormone it also may be high, indicating apparently a greatly increased solubility of $Ca_3(PO_4)_2$. Huge doses of vitamin D have a similar effect in animals and in man. The condition may be cured by removal of the tumors. A similar high calcium-low phosphorus ratio may occur in myelomatosis and malignant metastases to the bones.

Hypoparathyroidism, which may occur spontaneously or after parathyroidectomy, causes a fall in blood calcium and a rise in phosphorus. If the calcium falls to 7 mg. %, tetany ensues. It is relieved by the injection of calcium salts or parathormone or by administration of acids. Alkalies have the opposite effect. Vita-

min D is ineffective. (Tetany is not produced when the fall in calcium is due to protein depletion. Tetany may also be the result of alkalosis, without change in calcium; see section on acid-base balance.)

In *rickets* there is usually a low phosphorus, with an approximately normal calcium. Less often the phosphorus is about normal and the calcium is reduced. (See section on Rickets.)

In *osteomalacia* of adults, seen chiefly in pregnant women, the same conditions as in rickets appear to exist. In healing *fractures* phosphorus is often high, calcium about normal. High calcium has been reported in *polycythemia vera.* Low calcium often occurs in *chronic diarrhea.*

In advanced *nephritis with acidosis* there is a rise in phosphorus, even to 20 mg. %, with a reciprocal fall in calcium. It approximately parallels the rise in creatinine. Figures over 8 mg. % indicate impending uremia. Tetany may occur, but is rare.

High phosphorus also occurs in cases of *vomiting* which lead to marked chloride depletion.

There is a transient fall in phosphorus of about 1 mg. % after the *ingestion of glucose,* while the latter is being removed from the blood stream. This is attributed to a combination of glucose with phosphoric acid, which appears to be an essential step in the storage and utilization of glucose. This fall in phosphorus does not occur or is lessened or delayed in diabetes, and observations of blood phosphorus have been suggested to supplement those of the blood sugar in glucose tolerance determinations. A similar fall in phosphorus occurs after injections of insulin or epinephrin.

More information is obtained by a study of the total calcium and phosphorus metabolism than merely from blood analyses. This requires precisely regulated diets and quantitative estimations of calcium and phosphorus in both urine and feces over substantial periods and is impracticable except for research purposes.

Alkaline Phosphatase (Green-Bodansky). About 7 ml. blood are allowed to clot at room temperature. As soon as possible after coagulation, the tube is centrifuged and the serum (which must be clear and colorless) is pipetted off. The analysis should be made at once, but may be postponed a few hours without gross error if the serum is chilled in ice water. (The results are likely to be somewhat too high.) Gross errors are likely if the blood stands for some hours in a warm laboratory.

(1) Into tube 1 (blank) pipet 2 ml. serum, (2) add 4 ml. sodium-barbital solution, and (3) 1 ml. water. (4) In tube 2 (test) pipet 1 ml. serum, (5) add 2 ml. sodium barbital solution, (6) 5 ml. water, and (7) 1 ml. glycerophosphate solution. (8) Mix, stopper the tubes, and incubate in a water bath at 37° C. for just two hours. Both the time and the temperature must be accurately controlled. (9) Cool in ice water a few minutes. (10) Add 6 ml. 20 per cent trichloracetic acid to each and mix. (11) To tube 1 add 2 ml. glycerophosphate, and mix. (12) After 30 minutes filter through Whatman No. 30 filter paper. (13) Determine the phosphorus in the filtrates as for ordinary phosphorus determination (p. 782).

Calculation for blank if photoelectric colorimeter is used:

$$\text{Mg. \% P} = \frac{4 \times \text{Reading of filtrate} \times 3}{\text{Reading of standard} \times 2}$$

For blank if visual colorimeter is used:

$$\text{Mg. \% P} = \frac{4 \times \text{Reading of standard} \times 3}{\text{Reading of filtrate} \times 2}$$

For test (tube 2) with photoelectric colorimeter:

$$\text{Mg. \% P} = \frac{4 \times \text{Reading of filtrate} \times 3}{\text{Reading of standard}}$$

For test (tube 2) with visual colorimeter:

$$\text{Mg. \% P} = \frac{4 \times \text{Reading of standard} \times 3}{\text{Reading of filtrate}}$$

Phosphatase in "units" = (mg. % P in tube 2 minus mg. % P in tube 1) × .55

One "unit" is the amount of ferment which will liberate 1 mg. phosphorus from its organic combinations in one hour, under the conditions of the experiment.

If a high content of phosphatase is expected or found, the filtrate at step 13 should be sufficiently diluted with 10 per cent trichloracetic acid so that it will about match the color of the standard phosphorus solution in the subsequent determination, and the calculation adjusted accordingly.

REAGENTS: SODIUM BARBITAL SOLUTION. Dissolve 2.06 Gm. sodium barbital in 100 ml. water. Store in the icebox.

GLYCEROPHOSPHATE SOLUTION. Dissolve 3 Gm. sodium beta-glycerophosphate in 100 ml. water. Keep in the icebox. This keeps two to four weeks.

SIGNIFICANCE. Phosphatase is a ferment or group of ferments found in large amount in actively growing bone, which will split phosphate esters *in vitro* and which appears to bring about the deposition and absorption of calcium phosphate in bone. It is found in smaller amount in some other tissues and in the blood. The amount revealed by this method in normal blood ranges from 1.5 to 4.5 units. It is higher in children (5 to 14 units) than in adults. It is increased (from 3 to 10 times the normal) in generalized osteoporosis, in osteosclerosis, in hyperparathyroidism, and in the active stages of rickets and Paget's disease, even in cases in which the serum calcium and phosphorus are within normal limits. It may, therefore, be of considerable diagnostic value. An increase has also been reported in jaundice, particularly in obstructive jaundice. Phosphatase may also be increased (usually to a less degree) in myelomatosis and in metastatic carcinoma of bone.

Acid Phosphatase (Method of King and Armstrong (for Alkaline Phosphatase) Adapted by Gutman, Gutman and Robinson, 1940). (1) Into each of 2 test tubes (one marked "test," the other "control,") put 10 ml. buffered substrate solution, and put in water bath at 37° C. for five minutes. (2) To each tube add 0.5 ml. clear fresh serum or plasma free from any tinge of hemoglobin. (3) To the tube marked "control" add immediately 4.5 ml. diluted phenol reagent, mix and filter. (4) Stopper the tube marked "test," mix and put in water bath at 37° C. for exactly three hours. (5) Remove "test" tube from water bath, immediately add 4.5 ml. diluted phenol reagent, mix, and filter. (6) Transfer 10 ml. of each filtrate to clean test tubes. (7) In a third tube put 10 ml. mixed standard phenol solution and reagent (prepared as directed below). (8) To each tube add 2.5 ml. 20 per cent sodium carbonate solution, and put in water bath at 37° C. for five minutes, until color develops. (9) Remove from water bath, cool, and read in colorimeter.

If the color in the tube marked "test" is very dark, to get accurate results it is

necessary to repeat the entire test, using in the tube marked "test" 0.5 ml. of serum so diluted that it will give a color approximately like that in the standard tube. Due allowance must be made for this dilution in subsequent calculations. If, however, it suffices for purposes of diagnosis to know merely that the phosphatase is markedly increased, this may be omitted.

The result is expressed as units per 100 ml. of serum, one unit being the activity required to liberate 1 mg. of phenol under the conditions of the test.

Calculation, if a photoelectric colorimeter is used:

$$\text{Units of phosphatase per 100 ml.} = \frac{\text{Reading of test}}{\text{Reading of standard}} - \frac{\text{Reading of control}}{\text{Reading of standard}}$$

SOLUTIONS: BUFFERED SUBSTRATE. 0.005 M monophenyl phosphate in 0.1 M citrate, pH 4.9. This is prepared just before use by mixing equal parts of Solutions A and B. Recheck pH.

Solution A. Dissolve 1.09 Gm. disodium phenyl phosphate (Eimer and Amend) in 50 ml. water.

Solution B. Dissolve 42 Gm. crystalline citric acid in water, add 376 ml. normal sodium hydroxide and dilute to 1 liter. Adjust pH to 4.9 using nitrazine as indicator. Store both solutions in tightly stoppered bottles in the icebox.

PHENOL REAGENT. In a 1500-ml. flask dissolve 100 Gm. sodium tungstate and 25 Gm. sodium molybdate in 700 ml. water, and add 50 ml. 85 per cent phosphoric acid and 100 ml. concentrated hydrochloric acid. Boil for 10 hours with a reflux condenser, the stopper of which is wrapped with tin foil. Add 150 Gm. lithium sulfate, 50 ml. water, and a few drops of bromine and boil for 15 minutes without a condenser. Cool and dilute to 1 liter.

Before use dilute one part of solution with two parts of water.

ANHYDROUS SODIUM CARBONATE SOLUTION. Prepare a 20 per cent solution of anhydrous sodium carbonate.

STANDARD PHENOL SOLUTION. Dissolve 1 Gm. phenol crystals in N/10 hydrochloric acid to make a liter. To standardize: Put 25 ml. of this solution in a 250-ml. flask, add 50 ml. N/10 sodium hydroxide, and heat to 65° C. Add hot 25 ml. N/10 iodine solution, stopper flask, and let stand 30 minutes. Then titrate the excess of iodine with N/10 thiosulfate solution. Calculate from this figure how much the standard solution must be diluted to give a concentration of just 10 mg. in 100 ml. (The undiluted solution has about ten times this concentration.) One ml. N/10 iodine corresponds to 1.567 mg. phenol. Therefore if the solution is exactly correct, the 25 ml. of standard solution titrated will combine with 15.96 ml. of the N/10 iodine solution, and 9.04 ml. of N/10 thiosulfate will be required to neutralize the balance of the iodine. The volume to which 100 ml. of the strong standard must be diluted is 40 times the value of w in the equation $w = \frac{25 \times (25 - \text{No. ml. of thiosulfate used})}{15.96}$.

Store the diluted standard in the icebox. It keeps three months.

MIXED STANDARD PHENOL SOLUTION AND REAGENT. Prepare this daily by adding 5 ml. diluted standard phenol solution to 45 ml. diluted phenol reagent.

Acid phosphatase may also be determined by the Bodansky method by substituting the acid (citrate) buffer for the alkaline (sodium barbital) buffer. The figures obtained by the modified Bodansky procedure are different from (usually about half) those given by the Gutman method. As the reliability of the latter method is more firmly established, it must be regarded for the present as the method of choice.

In inexperienced hands, phosphatase determinations are apt to give erratic results. Close attention to details is essential, particularly with respect to the exact time and temperature of incubation. Hemolysis causes a misleading increase in acid phosphatase, whereas the activity diminishes on standing, particularly at room temperature.

Significance. Normal serum contains small amounts of acid phosphatase (derived from tissues other than the prostate), usually less than 3 units. Values from 4 to 6 units are border-line, from 6 to 10 usually significant, and above 10 always pathologic. A significant increase is practically limited to cases of carcinoma of the prostate in which bone metastases have taken place and is observed in from 75 to 85 per cent of such cases. Occasionally an increase may be found before clinical or roentgenologic evidence of metastases can be obtained. (Alkaline phosphatase may also be increased.) There is usually a marked fall following castration or the administration of estrogens in such cases, and this has been used as a guide to such treatment. A rise is very rare in any other diseases, including diseases such as Paget's and metastatic carcinoma (in bone) from other sources. The test is of no value for the early diagnosis of prostatic carcinoma.

Plasma Proteins

Procedure. Serum (or plasma) which must be free from hemoglobin is centrifuged until clear. Serum is preferable, unless fibrinogen is to be determined, as oxalate is somewhat disturbing.

Total Protein. Into a 50-ml. volumetric flask pipet 1 ml. serum (or plasma) and dilute to the mark with 0.9 per cent salt solution. Transfer 1 ml. diluted serum to a Pyrex digestion tube graduated at 35 and 50 ml. (Tube 1).

Albumin. All procedures must be carried out at incubator temperatures. To 1 ml. serum (or plasma) in a 50-ml. graduate add 30 ml. 22.2 per cent solution of anhydrous sodium sulfate, mix, stopper, and let stand in the incubator at 37° C. for three hours or more. Filter repeatedly through a fine grade of filter paper until perfectly clear. Transfer 1 ml. of filtrate to a Pyrex digestion tube (Tube 2).

Fibrinogen. If desired, this may also be determined. To 0.5 ml. plasma in a 50-ml. graduate add 48.5 ml. of 0.9 per cent salt solution and 1 ml. of 2.5 per cent calcium chloride solution. Mix and let stand about an hour. With a fine-pointed glass rod, detach the fibrin clot and transfer to a dry filter paper, squeezing out the fluid by rotating the rod. Transfer the clot to a Pyrex digestion tube (Tube 3).

Digestion. (1) To each tube, add 1 ml. 50 per cent sulfuric acid and a few chips of porcelain. (2) Heat to boiling over a micro-burner until the water has been driven off and dense white fumes appear. (3) Cover with a watch glass and continue heating, so that the mixture boils vigorously, continuing for four minutes. (4) Remove flame and allow to cool for one minute. (5) Remove the watch glass and with a fine pipet add 0.5 ml. saturated persulfate solution. (6) Replace burner and boil gently about one minute, until colorless. Repeat steps 4, 5, and 6, adding an additional 0.5 ml. of persulfate solution. (7) Cool and dilute to 35 ml. (8) Pipet 3 ml. standard nitrogen solution containing 0.15 mg. of nitrogen (as used in determining N.P.N.) into a 50-ml. volumetric flask; add 1 ml. of 50 per cent sulfuric acid, 1.0 ml. persulfate solution, and water to a volume of about 30 ml. (9) To the flask and each of the tubes add 3 to 5 drops of gum ghatti solution, and mix. (10) To each add 15 ml. Nessler's solution, and dilute the standard solution to the mark with water. (11) Mix and read in the colorimeter.

Before making the readings in the colorimeter, inspect the solutions carefully and discard if there is the slightest opalescence or turbidity. This difficulty can often be avoided by washing the solutions (following step 6) into 50-ml. volumetric flasks, diluting to about 30 ml., chilling them and also the Nessler's solution before nesslerizing (step 10), and by keeping the solutions whirling briskly in the flasks while rapidly pouring in the Nessler's solution from a graduate. The readings must be made without delay, preferably after about 5 minutes.

Determine the nonprotein nitrogen in the serum by the usual method.

Calculation, if a photoelectric colorimeter is used:

$$\text{Per cent protein} = \left[\left(\frac{\text{Unknown} \times 0.15 \times 100}{\text{Standard} \times \text{V}}\right) - \text{N.P.N.}\right] \times \frac{6.25}{1000}$$

With a visual colorimeter:

$$\text{Per cent protein} = \left[\left(\frac{\text{Standard} \times .15 \times 100}{\text{Unknown} \times \text{V}}\right) - \text{N.P.N.}\right] \times \frac{6.25}{1000}$$

V = volume of serum: 0.02 ml. in case of total protein, 0.0323 in the case of albumin, and 0.5 ml. for fibrinogen.

Total protein — albumin = globulin.

Albumin globulin ratio = albumin/globulin.

Determinations should be done in duplicate, including the globulin precipitation, which is the step at which error is most likely to occur.

As the fibrin from 1 ml. of normal blood contains from 0.4 to 0.6 mg. nitrogen, it may be necessary also to set up a second flask containing double the quantity of standard nitrogen solution in step 8.

Reagents: Sulfuric Acid. A 50 per cent by volume dilution is used.

Saturated Persulfate Solution. Put 6 to 7 Gm. potassium persulfate (nitrogen-free) in a bottle and add about 100 ml. distilled water. To insure saturation, some undissolved crystals should remain. This should be renewed every few weeks.

Saturated Sodium Sulfate Solution. Dissolve 22.2 Gm. anhydrous sodium sulfate in about 80 ml. water by heating; rinse into a 100-ml. volumetric flask, and dilute to the mark with water at 37° C.

Gum Ghatti Solution. See p. 767 for the preparation of this solution.

Other Reagents. The other reagents are those used in nonprotein nitrogen determinations.

Significance. There is from 0.25 to 0.4 per cent *fibrinogen* in normal blood plasma. It is formed solely in the liver. Its normal function appears to be limited to the production of blood coagulation. It is increased in most acute infections and inflammations, and in conditions in which there is destruction of body tissue. It is reduced in cases of extreme liver injury, but may be normal or even increased in cases showing extensive but not maximal degrees of destruction of liver tissue. It is, therefore, of little value as a test of liver function.

Normal plasma contains 7 per cent (6.3 to 8.0 per cent) of total protein, of which 4.4 per cent (3.8 to 5.2 per cent) is albumin, and 2.6 per cent (2.0 to 3.5 per cent) is globulin (including 0.2 to 0.4 per cent of fibrinogen). The normal albumin-globulin ratio is 1.7 (1.45 to 2.2).

It has been shown, particularly by Whipple and associates, that the plasma proteins constitute a part of the body store of protein, and can be withdrawn from the blood and utilized by the tissue cells in case of need. Injections of homologous plasma satisfy the needs of the cells completely and maintain nitrogen equilibrium in a starving animal. Conversely, a deficiency of plasma protein, such as may follow bleeding, may be restored by withdrawal of protein from the cells.

Another important function is to *control the distribution of fluid* between the capillaries and the intercellular tissue spaces. The plasma proteins do not pass

through the normal capillary endothelium (the intercellular fluids contain practically no protein), but exert a colloidal osmotic pressure or "oncotic" pressure (of 21 to 29 mm. Hg) which tends to draw fluid from the tissues into the vessels. This, it is believed, normally counteracts the effect of the hydrostatic pressure within the vessels, which tends to force fluid into the tissues. In the arterial end of the capillary loop the hydrostatic pressure prevails, and fluid passes into the tissues. In the venous end normally the colloidal osmotic pressure of the proteins is the greater, and the lost fluid is recovered. Albumin exerts about four times as great a colloidal osmotic pressure as globulin (per Gm.) and changes in it are of primary importance. Edema commonly occurs in any condition in which there is a substantial fall in plasma albumin. The critical point is 2.5 per cent albumin or 5.5 per cent of total protein (Peters and Van Slyke).

Fluctuations in total protein occur as a result of changes in plasma volume. A marked increase occurs with *dehydration,* as after protracted vomiting or diarrhea; in cholera, e.g., it may rise to 11 per cent. A slight decrease (to about 6 per cent) occurs in the first six months of pregnancy. A decrease occurs after *protein starvation* ("war edema") and in a great variety of wasting diseases. Marked decreases in albumin occur commonly in *"nephrosis,"* including amyloidosis, and in the active (nephrotic) stage of *hemorrhagic nephritis,* often falling to 2 per cent and even to less than 1 per cent. The globulin as a rule is not significantly reduced, and may even be increased. As a result the *albumin/globulin ratio falls* and may be reversed. One factor causing the low plasma protein is the loss of albumin in the urine, which in these conditions often amounts to 10 Gm. (up to 25 Gm. or more) per day. Malnutrition is also a factor.

Low plasma albumin and an inverted A/G ratio also occurs in advanced *disease of the liver.* There is much evidence that the plasma albumin (and possibly also the globulin) is synthesized in the liver.

The administration of *sodium chloride* markedly aggravates the edema, and rigid salt restriction helps to control it. Nothing is known as to the mechanism of this action. *Diets high in protein* (90 to 120 Gm. per day), even in cases of active nephritis, raise plasma albumin substantially (but usually not to normal) and often result in elimination of the edema and marked subjective improvement. These quantities do not appear to damage the kidney or lower renal function. The reserve protein of the body appears to be depleted in these conditions, and large amounts of protein may be taken and stored without increase in the nitrogen excretion in the urine. However, albuminuria, a lowered plasma albumin, and a tendency to edema persist, unless a spontaneous remission or recovery occurs.

Increase in globulin, especially fibrinogen, occurs in acute infections, including those associated with acute nephritis, and may partly mask the effect of the reduction in albumin. It is especially marked in kala-azar (q.v.). Globulin is also increased in carcinomatosis and especially in myelomatosis. This increase is a major factor in causing the accelerated sedimentation rate of the red cells seen in these conditions.

Edema may occur, *with normal plasma protein,* as a result of increased intracapillary pressure (local stasis, the chronic passive congestion of myocardial failure); or of damage to the capillary walls which permits protein to leak into the intercellular spaces and to neutralize the effects of the colloidal osmotic pressure of that left in the vessels (inflammatory processes, angioneurotic edema). It may also follow the administration of excessive amounts of alkali.

Cholesterol

Procedure. (1) Pour about 17 ml. solvent into a 25-ml. volumetric flask and add 1 ml. serum slowly while rotating the flask. (2) Bring to boiling in a water bath. Remove a moment and again bring to boiling. Remove and again reheat. (3) Cool in running water, bring to volume with solvent, mix, filter through S. and S. fat-free filter, and stopper. (4) Pipet 5 ml. filtrate into a Pyrex test tube and add a Hengar crystal. (5) In a second tube put 5 ml. working standard solution, containing 0.4 mg. cholesterol, and in a third tube 10 ml. standard solution. (6) Put tubes in a beaker of hot (nearly boiling) water, and let stand until the volume is reduced to about 0.5 ml. (but not dry). (7) Add 1 ml. chloroform, and evaporate until the residue appears to be just dry. (Do not overheat.) (8) Remove tubes from the beaker and cool. (9) Add 10 ml. chloroform to each, stopper loosely, and let stand five minutes in a water bath at 25° C. (10) Prepare reagent, and add 4 ml. to each tube at one-minute intervals. Mix and stopper. (11) Let stand just 10 minutes at 25° C. (12) Read in colorimeter, using light of 625 mμ wave length.

Calculation, if a photoelectric colorimeter is used:

$$\text{Mg. \% cholesterol} = \frac{\text{Reading of unknown} \times 200}{\text{Reading of standard}}$$

With a visual colorimeter, if the unknown is set at 20, the reading of the standard multiplied by 10 gives the cholesterol in mg. %.

Reagents: Solvent. Mix 3 parts dehydrated alcohol with 1 part anhydrous ether.

Chloroform (Reagent, Dehydrated).

Cholesterol Reagent. Prepare just before use. In an Erlenmeyer flask put 4 ml. acetic anhydride for each tube in the series plus 2 ml. While holding the flask under running water, add slowly 0.1 ml. concentrated sulfuric acid for each 1 ml. acetic anhydride.

Standard Solution, Stock. Dissolve 400 mg. cholesterol in 250 ml. chloroform in a volumetric flask. 1 ml. = 1.6 mg.

Working Standard Solution. Dilute 5 ml. stock standard solution to 100 ml. with chloroform. Store in the icebox in a glass-stoppered bottle. 1 ml. = 0.08 mg. 5 ml. = 0.4 mg.

Significance. The normal figures for cholesterol in whole blood and serum usually range from 150 to 250 mg. per 100 ml. Very little is known as to the function of cholesterol in the body. It is supposed to be concerned in the absorption and metabolism of fat. Although an alcohol and not a fat, it is classed as a blood lipoid (along with neutral fat, fatty acids, phosphatids, etc.), of which it normally constitutes about 20 to 40 per cent. It occurs partly as free cholesterol (40 to 60 per cent), and as cholesterol esters of fatty acids.

Blood cholesterol is little affected by the fat content of the *diet,* but it is increased by the administration of foods rich in cholesterol or in vegetable sterols. It is reduced in acute febrile *infections,* in most cases of *malnutrition* and cachexia,

and in severe *anemia* (although the blood fat may be high). In pernicious anemia it may fall to 70 mg. %. It is slightly high in pregnancy. Increases have been reported in cases of cholecystitis and gall-stones when *obstructive jaundice* is present, and in some cases of arteriosclerosis.

A marked *increase in cholesterol,* up to 350 to 600 mg. % (and also of fat), is frequent in *diabetes,* particularly in severe cases with a tendency to ketosis, falling rapidly under insulin treatment.

Marked increases of cholesterol (and all other lipoids) are seen in *"nephrosis"* and the nephrotic stage of active *nephritis.* In these diseases considerable cholesterol is excreted in the urine, and it constitutes the doubly refractile granules that may be seen in the renal tubular epithelial cells. In other types of nephritis, blood cholesterol is usually not increased. High cholesterol has also been reported in cases of generalized *xanthomatosis,* both in the diabetic and nondiabetic type, and in some cases of Christian-Schüller disease. It is much increased in *myxedema* (an important diagnostic point), and is diminished in hyperthyroidism.

Cholesterol Esters

Procedure. (1) Pipet into a small beaker 10 ml. filtrate prepared as for determination of cholesterol (steps 1, 2 and 3). (2) Add 1 ml. digitonin solution. (3) After a few minutes evaporate just to dryness over a water bath. (4) Add about 12 to 15 ml. petroleum ether and boil for a minute or two over a water bath. Decant the petroleum ether into a clean small beaker. In a similar manner extract the residue twice more with petroleum ether, adding these fractions to the second beaker containing the first petroleum ether extract. (5) Evaporate these combined extracts just to dryness over a water bath. (6) Add about 3 ml. chloroform to the residue and bring to gentle boiling. Decant into a 10-ml. glass-stoppered graduated cylinder. Repeat the extraction once or twice more with small amounts of fresh chloroform, but take care that the total volume of the three fractions does not exceed 5 ml. Cool, bring volume to the 5-ml. mark in the cylinder with chloroform. (7) In a second cylinder or tube put 5 ml. working standard cholesterol solution. (8) Proceed with steps 10, 11, and 12 in the determination of total cholesterol.

Calculation, using the photoelectric colorimeter:

$$\text{Cholesterol esters in mg. \%} = \frac{\text{Reading of unknown} \times 100}{\text{Reading of standard}}$$

With visual colorimeter:

$$\text{Cholesterol esters in mg. \%} = \frac{\text{Reading of standard} \times 100}{\text{Reading of unknown}}$$

Total cholesterol — cholesterol esters = free cholesterol.

In this test, the free cholesterol combines with the digitonin to form a compound insoluble in petroleum ether, whereas the esters are dissolved and removed.

Additional Reagents. *Petroleum ether* and a 1 per cent solution of *digitonin* in 95 per cent alcohol are used.

The esterization of the free cholesterol is believed to take place in the liver. Normally from 40 to 60 per cent of the total cholesterol is in the form of esters. In cases with liver injury, the total and relative amount occurring as esters may be diminished.

Quantitative Determination of Alcohol in Body Fluids

Bogen Method: METHOD IN BRIEF. Air is drawn through 1 ml. of specimen to be examined and then through 5 ml. of Anstie's reagent (dichromate-sulfuric acid) while both are immersed in a boiling water bath. The alcohol present is measured by comparing color of reagent with previously prepared standards containing known amounts of alcohol. The color varies from reddish yellow to greenish blue.

METHOD. Blood is collected by venepuncture and oxalated as for other chemical procedures (2 mg. oxalate per ml. of blood). When cleansing the arm with alcohol or tincture of iodine prior to puncture, the area is allowed to dry completely to avoid fouling the needle.

Four tubes are set up in a row and connected for aeration. In the tube farthest from the suction pump are placed 5 ml. of Anstie's reagent, to insure that no trace of alcohol or other volatile reducing substance is introduced with the air current (suggested by Engelfried). In the second tube are placed the following: 1 ml. of blood, urine, or other fluid to be examined, 1 ml. of Scott-Wilson reagent (for fixation of acetone), 3 to 5 ml. water, and 5 to 10 drops of liquid petrolatum (optional) to prevent foaming. The third tube is empty (safety tube) to prevent fluid foaming over directly into the reagent tube. The last tube nearest the suction pump contains 5 ml. of diluted Anstie's reagent. The connected tubes are immersed *very slowly* in a boiling water bath and aerated gently at moderate speed for 10 minutes. The color of the reagent tube is compared with that of known standards.

The method depends upon the fact that, when a solution of potassium bichromate in sulfuric acid is treated with alcohol, the alcohol is oxidized, largely to acetaldehyde, while the bichromate is reduced with the formation of chromic sulfate according to the following equation:

$$K_2Cr_2O_7 + 4H_2SO_4 + 3C_2H_5OH = 3C_2H_4O + K_2SO_4 + Cr_2(SO_4)_3 + 7H_2O.$$

The green color in the completed reaction is due to the production of chromic sulfate.

SCOTT-WILSON REAGENT. Dissolve 1 Gm. mercuric cyanide in 60 ml. distilled water, using a heavy-walled glass jar. Add a solution of 18 Gm. sodium hydroxide in 60 ml. water, stir vigorously with a heavy glass rod, add 0.290 Gm. silver nitrate dissolved in 40 ml. water. If turbid, allow to stand two or three days; decant the clear supernatant fluid. This keeps two months.

ANSTIE'S REAGENT. Dissolve with the aid of heat in water bath 0.666 Gm. potassium bichromate (C.P.) in 100 ml. concentrated sulfuric acid (C.P.). This will be labeled "Concentrated Anstie's" and used in preparing the standards. Dilute with equal parts distilled water for use in the test.

PREPARATION OF STANDARDS. Make a 1 per cent solution of alcohol by pipetting 1.260 ml. absolute alcohol at 15° C. into distilled water. Make up volume to 100 ml. in volumetric flask. Prepare a series of 11 tubes (12 by 150 mm.), labeled 0.0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0 mg. per ml., respectively. Pipet the 1 per cent alcohol into each tube to represent the above figures. Make up volume in each tube to 2.5 ml. with distilled water. Add to each tube 2.5 ml. concentrated Anstie's reagent (⅔ per cent potassium dichromate in sulfuric acid). Seal tubes by heating and drawing from top.

Example. Tube marked "0.5 milligram per milliliter" contains:

	Milliliters
1 per cent alcohol	0.05
Distilled water	2.45
Concentrated Anstie's reagent	2.50
Total	5.00

These standards are reliable for approximately three months, after which time the color in the higher concentrations of alcohol breaks down and begins to fade.

It has been our routine in persons who seem to have 3 mg. alcohol per ml. of blood or higher to repeat the test using only 0.5 ml. of blood and to multiply by 2 for the final reading. This has been considered advisable as matching of color, easy in the lower, is more difficult in the higher standards. One is also approaching the limit of capacity of the reagent at the 4-mg. concentration.

A small color change approximating the 0.5-mg. standard is, in our experience, produced by all normal bloods in the above method. This change is produced by some volatile organic constituent of the blood, probably, at least partially alcohol, for there is known to be alcohol present in minute quantities in the tissues normally. Therefore a change corresponding to the 0.5-mg. standard should not be taken to mean that the individual being examined has imbibed alcohol.

Nonvolatile material, glycerin, phenol, cresol, etc., added directly to the reagent, produce the same color change as alcohol. Ether as well as other volatile organic substances not encountered in the body fluids also reacts similarly. Therefore, care and cleanliness with instruments and glassware as well as in the performance of the test are essential to the integrity and reliability of the procedure.

Ether, however, will reduce Anstie's reagent only when the rate of aeration is very slow. Engelfried has shown that blood from patients anesthetized with ether, or to which measured amounts (1 to 3 mg. per ml.) of ether had been added caused no more reduction of Anstie's reagent than did normal blood.

Because of the reaction, with the reagent, of substances in cork and rubber stoppers, tubed standards, which are to be kept for some time and which may be inverted, should be sealed as directed above by heating and drawing out the glass.

Intoxication. In our experience the majority of men showing over 2 mg. alcohol per ml. of blood or urine had been pronounced intoxicated by the clinical examiner, acting independent of the laboratory. The vast majority below the 2-mg. level had in like manner been pronounced not intoxicated. Thus the 2-mg. concentration was considered the dividing line, as a rough approximation. In New York and a number of other states, however, 1.5 mg. per ml. of alcohol in the blood is regarded as prima facie evidence of intoxication in the case of motor-vehicle accidents. No person with less than 1 mg. of alcohol in 1 ml. of urine gave clinical evidence sufficient to justify a diagnosis of acute alcoholic intoxication. Above this concentration, the frequency of the clinical symptoms of drunkenness increased rapidly with the amount of alcohol found. Seventy-five per cent of men who showed blood alcohol concentrations of 1.5 mg. to 2.0 mg. per ml. were, by independent clinical examination, judged to be intoxicated. Eighty-five per cent of the men showing 2.5 to 3.0 mg. concentrations and all of those showing 3.5 to 4.0 mg. were judged intoxicated and 30 per cent were in coma. Death has occurred with concentrations of 4.5 to 5 mg. In experimental animals 6.0 mg. kills.

The susceptibility of the brain to alcohol varies little between one normal individual and another. The apparent variation in susceptibility in different individuals is thought to be governed more by the rate of absorption on one hand, and oxidation, exhalation, and excretion on the other, than to any inherent resistance of the cell protoplasm to the toxic effects of alcohol. Haggard and Greenberg (1937), however, found that the lethal concentration in the blood for rats

was higher (12 mg. per ml.) if the blood sugar was 200 mg. % than if it was low (8.0 mg. per ml. with a blood sugar of 70 mg. %).

Sulfonamides

Procedure for Free Sulfonamide (Bratton and Marshall). (1) Pipet 1 ml. oxalated blood into a 125-ml. Erlenmeyer flask, and (2) add exactly 13 ml. distilled water. (3) Shake and let stand five minutes, until completely laked. (4) Add just 6 ml. 10 per cent trichloracetic acid and mix thoroughly. (5) After five minutes filter through a No. 30 Whatman filter paper. (6) Pipet 10 ml. filtrate into a test tube. (7) Into three other test tubes pipet 10 ml. of each of the three working standard solutions. (8) Into each tube add 1 ml. 0.1 per cent solution of sodium nitrite. (9) After three minutes add 1 ml. 0.5 per cent ammonium sulfamate solution. (10) After two minutes add 1 ml. 0.1 per cent solution of N(1-naphthyl)ethylenediamine dihydrochloride and mix. (11) After five minutes read in colorimeter, using the standard tube which most nearly matches the filtrate.

Total Sulfonamide. Pipet 10 ml. filtrate prepared as above (steps 1 to 5) into a test tube graduated at 10 ml., add 0.5 ml. of 4 N hydrochloric acid, and heat one hour in boiling water bath. Cool, bring the volume just to 10 ml. with water, and proceed with the determination as in steps 7 to 11 above.

This hydrolysis liberates sulfonamide which may be present in conjugated form, so that it participates in the reaction. Conjugated sulfonamide yields no color.

It is necessary to know what particular sulfonamide has been administered, since there is no practicable way to determine this in the laboratory.

It is essential to use standard solutions made up with the same sulfonamide as that which is to be estimated in the blood, or else to multiply the result as calculated directly from the colorimeter readings by a correction factor which can be taken from Table 73. For practical clinical purposes the use of these conversion factors is recommended, particularly in small laboratories, since it eliminates the need for maintaining a large number of different standard solutions.

Reagents in Free Sulfonamide and Total Sulfonamide Determinations: Trichloracetic Acid. A 10 per cent solution is used.

Sodium Nitrite Solution. Dissolve 0.1 Gm. sodium nitrite in 100 ml. distilled water. Prepare freshly every day, or store in icebox (in which it will keep about a month).

Ammonium Sulfamate Solution. Dissolve 0.5 Gm. ammonium sulfamate in water and make up to 100 ml.

N(1-naphthyl)Ethylenediamine Dihydrochloride. Dissolve 0.1 Gm. in 100 ml. of water and store in brown glass bottle.

4 N Hydrochloric Acid. Dilute 171 ml. concentrated hydrochloric acid to 500 ml. with water.

Standard Solution: stock standard. Dissolve 0.2 Gm. sulfanilamide in 1 liter distilled water (volumetric flask). If a sulfadiazine standard is desired, it is convenient to use 0.2176 Gm. sodium sulfadiazine, diluting to 1 liter. Instead, one may put 0.2 Gm. sulfadiazine in a 1-liter volumetric flask and dissolve in a little water to which has been added 2 ml. 10 per cent sodium hydroxide. When solution is complete, dilute to the mark. Store in the icebox, in which it keeps for months.

working standard—strong. Pipet 5 ml. stock standard into a 100-ml. volumetric flask, add 27 ml. 10 per cent trichloracetic acid, and dilute to the mark. This contains 0.01 mg. drug per ml.

working standard—medium. Pipet 5 ml. stock standard solution into a 200-ml. volumetric flask, add 54 ml. 10 per cent trichloracetic acid, and dilute to the mark. This contains 0.005 mg. per ml.

working standard—weak. Pipet 1 ml. stock standard into a 100-ml. volumetric flask, add 27 ml. 10 per cent trichloracetic acid, and dilute to the mark. This contains 0.002 mg. per ml.

Calculation, if the weak standard is used, for the photoelectric colorimeter:

$$\frac{\text{Reading of unknown}}{\text{Reading of standard}} \times 4 = \text{sulfonamide in mg. \%}$$

For a visual colorimeter:

$$\frac{\text{Reading of standard}}{\text{Reading of unknown}} \times 4 = \text{mg. \% of drug}$$

If a stronger standard was used, for the factor 4 substitute 10 in case of the medium standard, and 20 in case of the strongest standard.

The same technical procedure is followed, regardless of which sulfonamide is to be determined. If, however, a sulfanilamide standard solution was used in determining the concentration of sulfadiazine (e.g., in blood) the figure obtained in the calculation above is multiplied by the factor 1.45. (See Table 73.)

Table 73

CONVERSION FACTORS FOR SULFONAMIDES

Drug Used as Standard	*Drug to be Determined*				
	Sulfanilamide	*Sulfacetamide or Sulfanylguanidine*	*Sulfapyridine or Sulfadiazine*	*Sulfathiazol*	*Sulfasuxidine*
Sulfanilamide	1.00	1.24	1.45	1.48	2.06
Sulfacetamide or Sulfanylguanidine	.80	1.00	1.16	1.19	1.66
Sulfapyridine or Sulfadiazine	.69	.86	1.00	1.02	1.43
Sulfathiazol	.67	.84	.98	1.00	1.38
Sulfasuxidine	.48	.60	.70	.72	1.00

After J. J. Engelfried.

Simplified Procedure (Engelfried). This method is recommended only when suitable laboratory facilities are not available, but the results are adequate for practical purposes.

(1) In a 100-ml. flask put 27 ml. 95 per cent alcohol, and add with a volumetric pipet 3 ml. oxalated blood. Stopper and shake occasionally for 10 minutes. (2) Filter, covering the funnel to prevent evaporation. (3) Into one test tube pipet 10 ml. filtrate, and into a second tube 1 ml. working standard solution and 9 ml. 95 per cent alcohol. To each tube add (4) 1 ml. N hydrochloric acid in alcohol, and shake. (5) Add a piece of nitrite paper 3 × 5 mm. in size, and shake occasionally for three minutes. (6) Add a piece of dye paper 10 × 25 mm. in size, and shake occasionally for 10 minutes. (7) Read in colorimeter or, if this is not available, into a test tube put 5 ml. of solution from the standard tube, and into a second test tube of the *same diameter*, put 5 ml. of solution from the unknown. Compare the colors.

If they match exactly, the blood contained 5 mg. sulfonamide per 100 ml.

If one is darker, dilute with 95 per cent alcohol until it just matches the paler tube, noting the precise amount of alcohol used.

Calculation, if the color of the unknown was darker than the standard:

Sulfonamide in mg. per 100 ml. = 5 plus number of ml. of alcohol added in the dilution.

If the color of the standard was the darker:

$$\text{Sulfonamide in mg. \%} = \frac{5}{\left(\frac{\text{5 plus ml. of alcohol used for dilution}}{5}\right)}$$

Multiply this figure by the proper conversion factor, if the sulfonamide to be determined is different from that used in the standard solution.

REAGENTS: ALCOHOL. Ninety-five per cent alcohol is used.

N HYDROCHLORIC ACID IN ALCOHOL. Add 10 ml. concentrated hydrochloric acid to 110 ml. of 95 per cent alcohol.

NITRITE PAPER. Saturate ordinary filter paper in a 10 per cent solution of sodium nitrite. Dry, and cut into pieces 3 × 5 mm. in size. If stored in colored-glass bottles it keeps a year or more.

AZO DYE PAPER. Saturate filter paper with a 1 per cent aqueous solution of N(1-naphthyl)ethylenediamine dihydrochloride. Dry and cut into pieces 10 × 25 mm. in size.

STOCK STANDARD SOLUTION. If pure drug is not available, dissolve a 0.5-Gm. tablet in water and dilute to 1 liter.

WORKING STANDARD SOLUTION. Pipet 10 ml. stock standard solution into a 100-ml. flask and dilute to volume.

Thiocyanates

Procedure (Barker). (1) Transfer 5 ml. 10 per cent trichloracetic acid solution to a test tube and add 5 ml. clear serum or plasma. Shake. (2) After 15 minutes filter through a small filter or centrifuge at high speed. (3) Pipet 5 ml. clear filtrate into a test tube and add 1 ml. ferric nitrate reagent. (4) Into three other tubes pipet 5 ml. of each of the three working standard solutions. (5) To each of these tubes add, with a volumetric pipet, 5 ml. 10 per cent trichloracetic acid and 2 ml. ferric nitrate reagent. (6) Read in colorimeter, using the standard which most nearly matches the color of the filtrate.

Calculation, with a photoelectric colorimeter:

$$\text{Thiocyanate in mg. \%} = \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times 10$$ if the strong standard is used. For the factor 10, substitute 7 if the medium standard is used, or 4 for the weak standard.

REAGENTS: TRICHLORACETIC ACID, 10 PER CENT SOLUTION. Dissolve 10 Gm. trichloracetic acid in 100 ml. distilled water.

FERRIC NITRATE REAGENT. Dissolve 50 Gm. ferric nitrate crystals in about 500 ml. distilled water in a 1-liter volumetric flask; add 25 ml. concentrated nitric acid, dilute to the mark and mix.

SILVER NITRATE SOLUTION. Dissolve 2.9195 Gm. silver nitrate in water in a 1-liter volumetric flask; add 5 ml. of nitric acid, dilute to the mark and mix. Store in a brown bottle in the dark.

POTASSIUM THIOCYANATE SOLUTION—STOCK. Dissolve 1.25 Gm. potassium thiocyanate in 1 liter water. Titrate as follows: Pipet 20 ml. silver nitrate solution into a small porcelain dish, add 5 ml. nitric acid and about 0.3 Gm. powdered ferric ammonium sulfate. From a buret add the thiocyanate solution, stirring constantly, until a salmon-red color is obtained that persists for 15 seconds. If necessary dilute the thiocyanate solution until exactly 20 ml. neutralizes 20 ml. silver nitrate solution. This should be checked every six months.

POTASSIUM THIOCYANATE—WORKING SOLUTIONS. *Strong Standard.* Dilute 100 ml. stock standard to 1 liter. Five ml. contains 0.5 mg. thiocyanate ion.

Medium Standard. Dilute 70 ml. stock standard to 1 liter. Five ml. contains 0.35 mg. thiocyanate ion.

Weak Standard. Dilute 40 ml. stock standard to 1 liter. Five ml. contains 0.2 mg. thiocyanate ion.

Significance. This test is useful in controlling the administration of thiocyanates in the treatment of hypertension. The optimum concentration varies to some extent with individual patients, but is usually from 6 to 12 mg. %. Concentrations of 15 mg. % or higher indicate excessive dosage and are likely to be associated with symptoms of intoxication.

Bromides

The method of Wuth (1927) as modified by Katzenelbogen and Czarski (1934) affords a simple and quick estimation of the quantity of bromides in the blood, which is useful in detecting intoxication from overdosage with the drug and in controlling treatment.

Procedure. (1) Add 1 ml. clear serum to 2.6 ml. 5 per cent trichloracetic acid in a small test tube, mix, and filter through a small filter. (It may be filtered immediately; no bromide is lost in the precipitate.) (2) Transfer 1 ml. filtrate to a comparator tube about 10 mm. in diameter and add 0.2 ml. 0.5 per cent gold chloride solution. (3) Compare at once with the series of standard tubes, previously prepared.

Standard Tubes. Prepare a stock solution containing 0.5 per cent sodium bromide in 0.6 per cent salt solution. Into each of a series of 12 test tubes put quantities of stock bromide solution, beginning with 0.5 ml. in the first tube and increasing the quantity by 0.5 ml. in each succeeding tube, to 6.0 ml. in the twelfth tube. Make two additional tubes containing 7 ml. and 8 ml. respectively. Add enough 0.6 per cent salt solution to each tube to bring the volume to 10 ml. and mix. These correspond to serum bromide concentrations ranging from 25 mg. per 100 ml. in the first tube to 300 mg. in the twelfth and 400 mg. in the fourteenth tube.

Put 1 ml. of each of these dilutions in a series of comparator tubes identical in diameter with that used above, and add to each 2.6 ml. 5 per cent trichloracetic acid and 0.72 ml. of 0.5 per cent gold chloride solution; mix.

These standard tubes are semipermanent, in that they will keep their color for from one week to six months if they are covered with paraffin oil, tightly stoppered and kept in the dark. If it is desired only to determine the existence and approximate degree of bromide intoxication, it suffices to prepare four dilutions, containing 2, 3, 4 and 6 ml. stock solution, each diluted to 10 ml., corresponding to 100, 150, 200 and 300 mg. per 100 ml. of blood.

Normal serum gives no color. A reading over 150 mg. per 100 ml. indicates overdosage and is often associated with symptoms of intoxication.

Bromides may be detected in the urine by shaking up with animal charcoal, filtering, and adding to 5 ml. filtrate 1 ml. 20 per cent trichloracetic acid solution and 1 ml. 0.5 per cent gold chloride solution. If no brown color appears, a test of the blood is useless.

Diastase

This may be determined by Winslow's method (p. 840), substituting 1 ml. of serum or whole blood for urine. Normal figures are from 8 to 64 units. The quantity is increased in diabetes mellitus, in nephritis with renal insufficiency, and markedly in severe pancreatic disease.

Disturbances of Acid-base Equilibrium

The blood plasma and other body fluids have a slightly alkaline reaction. The normal metabolic processes result in the continuous production of acid end-products which tend to make the reaction of the blood more acid. The physiologic activities of the body, however, are so adjusted that they keep the variations in

the reaction of the plasma within narrow limits. This is accomplished first, by the immediate virtual neutralization of these acid substances as they are formed in the tissues, and second, by the elimination of the excess of acid radicals by the lungs and kidneys. Following the ingestion of unusual amounts of acid or alkali or as a result of disease this neutralizing mechanism may be so overtaxed, or its activities so disturbed, as to result in a significant shift in the reaction of the blood. An acidosis, or less often an alkalosis, is the result.* Any marked shift in the reaction is fatal if not quickly corrected or compensated. Such shifts are associated with characteristic clinical symptoms and with changes in the blood, urine, and expired air which make possible their early recognition. To recognize these conditions and treat them intelligently it is essential to be familiar with the mechanisms which normally maintain this reaction at so nearly constant a level, and the usual ways in which it may be disturbed.

The strength (intensity) of an acid in solution, as distinguished from its quantity, or titratable acidity, depends upon the degree to which it is dissociated into hydrogen ions (H^+) and acid ions (A^-). That is, it depends upon the *hydrogen ion concentration* of the solution. This is expressed in terms of normal acidity, as a fraction of which the numerator represents the quantity of dissociated H ions present, and the denominator the quantity of H in a normal solution (1 Gm. per liter). In the case of a strong acid like hydrochloric acid, which is almost completely dissociated in dilute solution, the reaction (H^+ concentration) of a one hundredth normal solution would be nearly 0.01 N. In case of a weak acid like carbonic acid the H^+ concentration of a 0.01 N solution would not be 0.01 N, but very much less than this (circa 0.00007 N) because only a small fraction of its H is ionized. In pure water, which is dissociated to a slight extent into H^+ and OH^- ions, and which is neutral because the number of H^+ and OH^- ions are equal, it has been determined that the H^+ concentration is 0.0000001 N, or 1×10^{-7}. To avoid the use of clumsy fractions it is customary to express the reaction of such solutions in terms of the negative logarithm of the fraction, which is indicated by the *symbol pH*. Thus the pH of water is $-\log (0.0000001), = -\log (1 \times 10^{-7}) = -(-7), = 7$, the neutral point. More acid solutions have a higher H^+ concentration, and a smaller negative log (pH). The pH of a 0.01 N solution of hydrochloric acid, having a H ion concentration of (nearly) 0.01 N, would be $-\log (0.01), = -\log (1 \times 10^{-2}), = -(-2)$, or 2. Alkaline solutions have a higher pH than 7. Thus the pH of a 0.01 N solution of sodium hydroxide would be approximately 13.

The *pH of the blood* and tissues usually ranges from 7.3 to 7.5, and the maximum range compatible with life is from 7.0 to about 7.8.

The possibility of maintaining the reaction of the blood within such narrow limits depends upon the presence of *buffers* in the blood. A buffer is a substance which lessens the change in reaction which follows the addition of acid or alkali to a solution, as compared with the change which would occur if no buffer were present. All buffers are mixtures of a weak acid and its basic salts, or a weak base and its acid salts. The buffers in the blood which are of chief importance are carbonic acid and its bicarbonate salts, and hemoglobin (a weak acid at pH 7.4) and its basic salts. The plasma proteins have some buffer action. The monobasic

*The term "acidosis" is a misnomer, since during life the body fluids never acquire an acid reaction. There is at most only a shift toward the neutral point. We retain the term, however, because it is in general use.

and dibasic phosphates also constitute a buffer, but their quantity is too small to exert an appreciable effect in the blood.

The *mechanism of the action of a buffer* may be best explained by an example (from Peters and Van Slyke). If enough hydrochloric acid is added to a neutral unbuffered solution to give a concentration of 0.05 N, the H^+ concentration will be nearly 0.05 N, and the pH will be lowered from 7.0 to about 1.3, a drop of 5.7 units. If instead it is added to a solution containing both carbonic acid and sodium bicarbonate in 0.1 M concentration, the following reaction will take place: $HCl + NaHCO_3 = NaCl + H_2CO_3$. The strong acid will be replaced by a corresponding amount of the weak acid, H_2CO_3, while half the $NaHCO_3$ is used up. The ratio $NaHCO_3/H_2CO_3$ will fall from 1/1 to 0.5/1.5, and the pH will fall (the "neutralization" of an acid by a buffer is not complete), but only from 6.1 to 5.3, or 0.8 unit.

In the blood, carbon dioxide exists in two forms; partly combined as bicarbonates of the blood bases (chiefly sodium), and partly free as carbon dioxide or carbonic acid in physical solution in the plasma. Normally the ratio of combined carbon dioxide to free carbon dioxide is about 20 to 1.

The *reaction of the blood* (*pH*) depends directly on the ratio between the bicarbonate content of the plasma and its carbon dioxide tension (the amount of carbon dioxide in physical solution).

This can be calculated, if the values of these two factors are known, from the Henderson-Hasselbalch equation, which in its simplified form is given by Peters and Van Slyke as follows:

$$pH = 6.10 + \log \frac{BHCO_3}{H_2CO_3} = 6.10 + \log\left(\frac{mM(CO_2) - 0.0301p}{0.0301p}\right).$$

In this formula $mM(CO_2)$ represents the total CO_2 (free and combined) in the plasma, expressed as mM; and p the CO_2 tension of the plasma in mm. of Hg. Numerical values determined by calculation from experimental data have been substituted for pK′ and a in the original equation. (These values are valid only for serum or plasma at 38° C.)

It is obvious that if any two of these three factors, pH, CO_2 content, and CO_2 tension, can be directly measured, the third can be calculated from the equation, or more simply from nomographic line charts which have been constructed according to the terms of the equation. Of these the CO_2 content is the most readily determined, by gas analysis (p. 808). The pH can be determined directly by the colorimetric method of Cullen (1922), or one of its modifications. The CO_2 tension is more difficult, but it can also be determined directly by the method of Van Slyke et al. (1932) by measuring the CO_2 tension in a small bubble of air which has been brought into equilibrium with 9 volumes of whole blood. It can be calculated by Eisenmann's method (1926) from determinations of both the CO_2 content of the plasma (or serum), and the CO_2 combining power of plasma when saturated with CO_2 at two or more different tensions. It may also be determined indirectly by measuring the CO_2 tension of the expired air (by the Haldane or Fridericia methods, without rebreathing), which is usually identical with the tension in arterial blood. Details of these procedures will not be given as they require equipment available only in large laboratories in which special textbooks on the subject will be available.

The amount of *combined CO_2* (bicarbonate) present depends directly on the amount of base in the blood which is not combined with stronger acids. If base is liberated when such an acid is eliminated by combustion or excretion, it immediately combines with free CO_2 in the plasma. As a result the ratio

$BHCO_3/H_2CO_3$ rises and the pH is slightly increased. If free acid is produced or absorbed, it immediately robs some of the bicarbonate of its base, which neutralizes the acid, and increases the free CO_2 in the plasma. The bicarbonate/CO_2 ratio is lowered, and the pH falls. This effect is largely counteracted by prompt elimination of the excess of CO_2 by the lungs, but some reduction in the plasma bicarbonate remains. Because the plasma bicarbonate is the most immediately available and quantitatively the most abundant store of base which can be utilized for neutralization of acids, it is commonly spoken of as the *alkali reserve* of the blood. Simple methods are available for its estimation. This alone does not suffice to determine the pH, but in the commoner types of disturbance of acid-base equilibrium, as in diabetes and nephritis, changes in pH usually follow, roughly, changes in alkali reserve. Although there are exceptions which will be discussed later, determination of the alkali reserve remains the most valuable single practicable procedure for the estimation of these disturbances.

The *quantity of CO_2* normally present as bicarbonate in the plasma of oxygenated blood (pH of 7.4, CO_2 tension of 40 mm.) is about 24 to 25 mM or 56 vol. %. (The total CO_2 is about 1.2 mM, or 2.7 vol. % higher. 1 mM CO_2 = 22.26 ml., or 2.226 vol. %.) The maximum normal range for total CO_2 is usually given as from 55 to 75 vol. %. The figures for whole blood are about 20 per cent lower, as there is less bicarbonate in the cells than in the plasma. The terms *acidosis* and *alkalosis* are commonly used to include conditions in which there is a significant alteration in the alkali reserve even though there is little or no change in the pH.

The amount of *free CO_2* present in the plasma depends directly upon the concentration or tension of CO_2 in the atmosphere with which it is in equilibrium. This depends upon the balance between its rate of production in the tissues and its rate of excretion in the expired air. Since the rate of production of CO_2 is not susceptible to direct control, maintenance of a normal CO_2 tension (and a normal pH) must depend primarily on the ventilation rate of the lungs. This is accelerated when CO_2 tension is increased, as by breathing air to which CO_2 has been added, and is usually diminished when CO_2 tension is lowered. However, it appears to be not the increased CO_2 tension itself, but the lowered pH which usually accompanies it, which causes the increase in ventilation rate, presumably by stimulating the respiratory center.

The *hemoglobin* of the red cells, with its basic salts, is a buffer second in importance only to the CO_2 and bicarbonate of the blood. It is of chief importance in minimizing the changes in reaction which occur with changes in the CO_2 content of the blood. This action is in part that of a simple buffer. When the CO_2 content (and tension) of the blood is increased in its circulation through the tissues, it becomes in effect a stronger acid, and robs the hemoglobin of a small part of the base combined with it, to form bicarbonate. This tends to make the bicarbonate/CO_2 ratio and the pH higher than it would have been had no hemoglobin been available. However, the *changes in the intensity of the acidity of hemoglobin* which occur with oxygenation and reduction far outweigh in their neutralizing effect its action as a simple buffer. As arterial blood circulates through

the tissues it simultaneously gives up O to the tissues and receives CO_2 from them (in about the proportion of 10 to 8). When hemoglobin is reduced by loss of O, it becomes less strongly acid, and as a result liberates base, which is sufficient to neutralize (by forming bicarbonate) about five-eighths of the CO_2 received, without any change in pH. The acidifying effect of the remaining three-eighths of the CO_2 is largely offset by the buffer action of the hemoglobin and the other buffers, so that with an average increase of 5 vol. % in the CO_2 content of the venous blood, the pH falls only about 0.02 point.

The bicarbonate thus newly formed within the red cells raises the ratio of bicarbonate/Cl in the cells to a point above that in the plasma. As a result there is a diffusion of $—HCO_3$ ions from the cells into the plasma and of —Cl ions from the plasma into the cells until the ratios are equalized (*Donnan's equilibrium*). The basic ions (in the red cells chiefly potassium) cannot pass through the cell membrane. This passage of $—HCO_3$ ions into the plasma equalizes the bicarbonate/CO_2 ratio, and therefore the pH, in the cells and plasma, and thus makes the buffer effect of the hemoglobin available to the whole blood, and not merely to the intracellular fluid, as would be the case if this diffusion could not occur. Incidentally, with the liberation of base by the hemoglobin and the simultaneous diffusion of Cl into the cells, the osmotic pressure within the cells rises, and water is drawn from the plasma into the cells, causing them to swell, until osmotic equilibrium is restored.

In the lungs the reverse process takes place. The hemoglobin after oxygenation becomes more strongly acid, and abstracts base from much of the bicarbonate present, liberating a corresponding amount of CO_2. This immediately diffuses out into the air in the alveoli until equilibrium is established, usually at a tension of about 40 mm. of Hg (2.7 vol. %, 1.2 mM per liter). There is a simultaneous passage of Cl and water from the cells to the plasma and some shrinkage of the cells. The same phenomena occur when venous blood is exposed to the air *in vitro*, except that the blood loses all its CO_2. It is obvious that in severe anemia the buffer action of the blood is reduced, and acidosis more readily established.

In addition to CO_2, *other acids* are produced in the course of normal metabolism. Some phosphoric and sulfuric acid are formed by the catabolism of protein. After violent exertion lactic acid is produced in the muscles, and if the diet is high in fat and low in carbohydrate, substantial amounts of ketone acids are produced. These are immediately neutralized by base from the bicarbonate and other buffers, as above described, and notable lowering of the pH is prevented by elimination of the liberated CO_2 by the lungs.

The *total amount of acid* which can be buffered by the blood in this way before an intolerable degree of acidosis is reached is substantial. Peters and Van Slyke estimate that to reduce the pH of 1 liter of blood from 7.4 to 7.0, about 28 milliequivalents of acid will be consumed. Of this about 18 mE are buffered by the bicarbonate alkali, 8 mE by the hemoglobin alkali, and 2 mE by the alkali of other buffers, chiefly the plasma proteins. As the total blood volume is somewhat over 5 liters, it would be able to buffer about 150 ml. of N acid. The total buffer power of the body tissues has been estimated to be about five times that of the total blood, or about 750 ml. additional N acid. Both together are equivalent to about 15 ml. of N acid per kilogram of body weight. Much of this neutralizing power depends upon the elimination of the CO_2 liberated by the bicarbonate, and is not merely the result of simple buffer action.

This neutralization is accomplished only at the cost of lowering the alkali reserve. To prevent dangerous depletion of the alkali reserve it is essential that the acid radicals be eliminated without losing a corresponding amount of base. With rest and adequate O supply the lactic acid is speedily oxidized to CO_2 and water, or is resynthesized into glycogen, and the ketone acids may be largely or entirely oxidized, the base so liberated remaining in the plasma as bicarbonate. The maintenance of a normal alkali reserve, however, depends mainly upon the *selective excretory capacity of the kidneys.* The reaction of the urine is normally much more acid than that of the blood. This is due to the ability of the kidney to excrete weak acids (hydroxibutyric) and acid salts, and hold back a large part of the base with which they are combined in the plasma. Normally the acid phosphates are the most important vehicle for this acid elimination. In the blood about 80 per cent of the phosphate is in the form of dibasic phosphate. In strongly acid urine nearly all is excreted as monobasic phosphate, so that nearly half the base required to neutralize the phosphoric acid while it was in the blood is retained as bicarbonate and replenishes the alkali reserve. If considerable amounts of ketone acids are present in the blood (always combined with base), as in diabetic acidosis, they are excreted in the urine and as much as 50 per cent of that excreted may be as free acid. The total amount of unneutralized acid (*titratable acid*) so excreted daily varies greatly, but normally is equivalent to 20 to 40 ml. of N acid. In severe acidosis it may equal 150 ml. of N acid.

If in spite of this acid excretion the blood bases begin to be depleted, the kidney begins to exercise its second important function in preventing loss of base. It breaks up urea with the *production of ammonia,* and utilizes this ammonia instead of fixed base for the neutralization of much of the acid radicals which it excretes. Even strong acids like hydrochloric acid and sulfuric acid may be excreted as ammonium salts. The normal daily excretion is about 30–50 ml. of N NH_3, and in severe acidosis it may rise to 500 ml. Ammonia cannot be utilized for the neutralization of acids in the body fluids. Only minute traces can be demonstrated in the blood.

The sum of the titratable acid + ammonia measures the excess of fixed acid over fixed base excreted, and serves as a rough measure of the bicarbonate content of the plasma in diabetic acidosis.

If, on the other hand, there is an excess of fixed bases in the blood, which rarely occurs except after ingestion of alkalies, the titratable acid and ammonia diminish or disappear, and bicarbonate may be excreted in the urine. The urine may then become a little more alkaline than the blood.

Disturbances of acid-base equilibrium may depend primarily upon changes in the quantity (tension) of CO_2 in the blood (disturbances of the ventilation rate), or upon disturbances of the relation of the blood bases to acids other than carbonic. The causes of the disturbances in these two groups of conditions are entirely different, as is the treatment. An understanding of these conditions is facilitated by a study of the illustration on p. 803, which is taken from Van Slyke.

Normally the pH of the blood is automatically kept at a nearly constant level

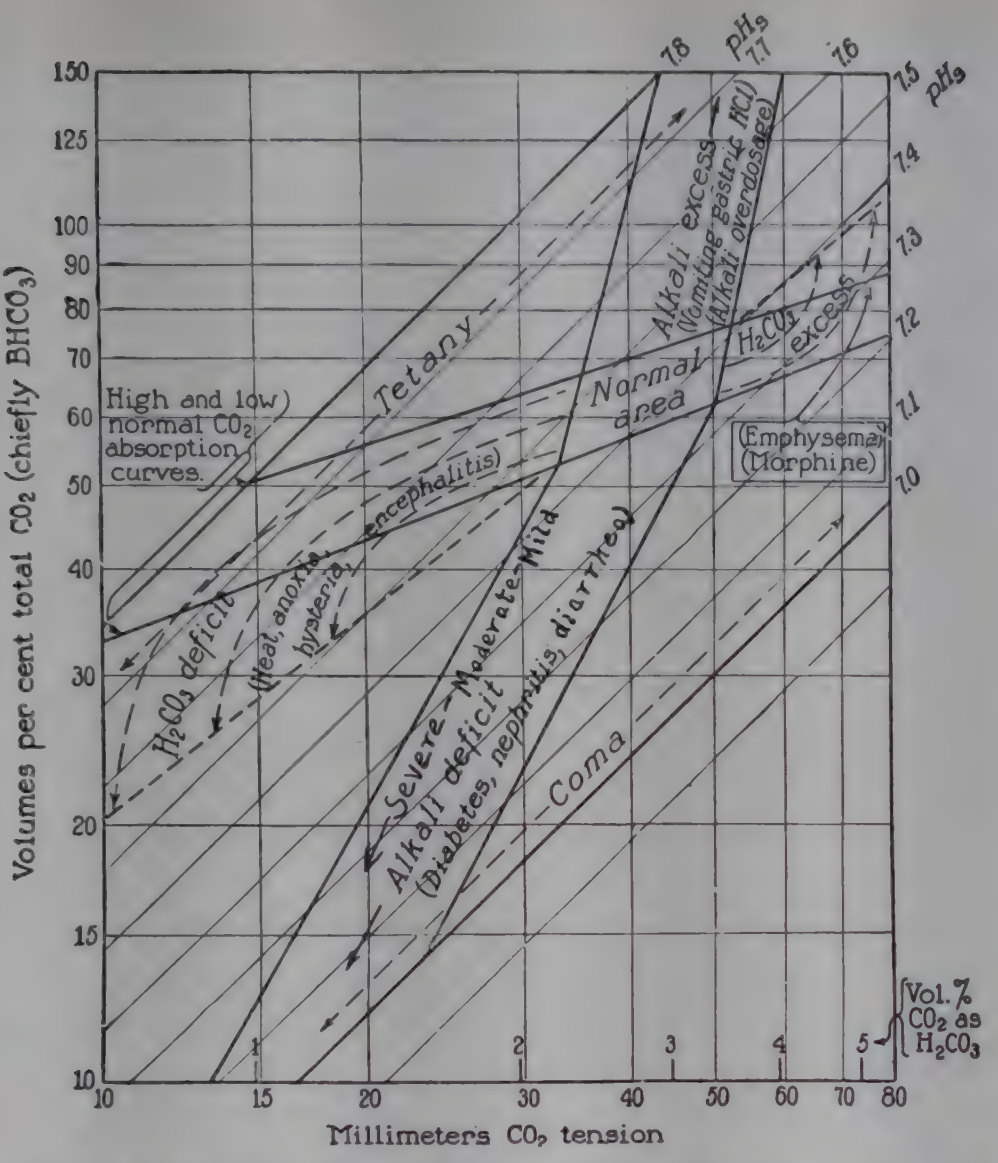


Chart showing changes in CO_2 content, H_2CO_3 content, CO_2 tension, and pH of the blood plasma in conditions of carbonic acid excess and deficit and of alkali excess and deficit. (Courtesy, D. D. Van Slyke and *Bull. N. Y. Acad. Med.*)

by changes in the ventilation rate of the lungs, which are brought about by the action on the respiratory center of slight changes in the pH of the blood. Thus a fall in pH stimulates the center, increases the ventilation rate, lowers the CO_2 tension, and raises the pH. If other factors interfere with this regulatory process, a shift in the reaction will occur. Thus a *diminished ventilation rate* may occur if the sensitiveness of the respiratory center is reduced by morphine, or if breathing is disturbed mechanically, as in emphysema or obstruction of the respiratory passages. Then the CO_2 tension rises and the pH falls. There is a *primary CO_2 excess.* This is partly compensated by an increased excretion of acid radicals (PO_4, Cl, etc.) and ammonia in the urine. The base so liberated neutralizes some of the excess of CO_2, raises the $BHCO_3/CO_2$ ratio, and partly corrects the fall in pH. This results in an increase in the plasma bicarbonate, in spite of the presence of some acidosis. Treatment aimed directly and solely to correct the acidosis in these conditions is not necessary, as death from lack of O will occur before the acidosis becomes dangerous in itself. Administration of alkali is not indicated. Dyspnea on exertion is the chief symptom.

Hyperventilation of the lungs reduces the CO_2 tension and raises the pH, even to the point of causing tetany (7.6+). There is a *primary CO_2 deficit*. This may occur as a result of breathing air with a low O tension (high altitudes), of hot baths, fever, hysteria, certain organic nervous diseases, and even by voluntary effort. A similar disturbance (high pH, low bicarbonate) has been reported after deep radiation therapy. It is partly compensated by arrest of renal excretion of acid and ammonia and by the increased excretion of fixed base. This reduces the alkali reserve, despite the presence of alkalosis. The logical treatment is to reduce the sensitiveness of the respiratory center by morphine, and to give salt and large amounts of fluid. In cases of tetany due to alkalosis there is no significant change in the blood calcium.

A *primary alkali excess* may occur as a result of overdosage with alkali, or loss of HCl from protracted vomiting (or gastric lavage) as in pyloric obstruction. The ratio $BHCO_3/H_2CO_3$ rises, and with it the pH. A plasma CO_2 content of 122 vol. % has been reported in pyloric obstruction. If the disturbance is marked, tetany may occur. The condition is compensated in part by slowing the respiration and raising the CO_2 tension, and in part by a lessened excretion of acid and ammonia, and by the excretion of sodium bicarbonate in the urine. In cases due to vomiting, dehydration and loss of electrolytes may be more serious than the alkalosis, and but little base is excreted. Treatment consists in overcoming the dehydration and re-establishing the urine excretion by injections of hypertonic salt solution. The kidney is then able to excrete the excess of alkali, and administration of acid is unnecessary.

The differentiation of an alkalosis due to primary alkali excess from an acidosis due to primary CO_2 excess cannot be made simply from an estimation of the alkali reserve, since this is increased in both conditions. If it depended solely on laboratory procedures it would be necessary also to determine either the pH or the CO_2 tension. Practically this is rarely necessary, as the conditions can usually be differentiated on clinical grounds alone.

A *primary alkali deficit* is the most common and most important type of disturbance of acid-base equilibrium. It may be due directly to loss of alkali in the stools after severe diarrhea, as in cholera and in infantile diarrheas. It can be produced by the administration of free acid or acidifying salts. The similarity of the symptoms of dogs poisoned with acid (particularly the dyspnea) to those of patients in diabetic coma led to the recognition of acidosis as the cause of the latter. It occurs temporarily after severe exercise. It may occur in diabetes, and in the acute ketoses of children as a result of retention of ketone acids. It occurs in starvation, and in cases on a ketogenic diet. In conditions associated with vomiting but without much loss of free hydrochloric acid, a ketosis with alkali deficit commonly develops. In children this may be far more intense than in case of simple starvation. A fall in pH and in plasma bicarbonate occurs during general anesthesia. It may occur (most markedly in venous blood) in circulatory failure, although conditions are complicated by varying degrees of CO_2 excess.

It may occur in advanced renal insufficiency as a result of loss of selective secretory power, so that less acid is excreted and more fixed base escapes.

In all these conditions the distinctive feature is the *lowered plasma bicarbonate* and lowered pH. The mechanism of compensating for this disturbance has already been discussed: hyperpnea with a lowering of the CO_2 tension; excretion of acid salts and even free hydroxibutyric acid in the urine; and the substitution of NH_3 for fixed base in the salts excreted. These conditions are usually associated with a profuse diuresis which may lead to a dehydration and salt depletion which is almost as serious as the acidosis. *Treatment of diabetic acidosis* consists first in the administration of insulin, usually with glucose, to secure combustion of the ketone acids; and, second, the administration of salt solution to restore salts and fluid and maintain urine secretion. The base liberated by the oxidation of the ketone acids largely replenishes the alkali reserve, and if renal function is good, any remaining excess of acid can be excreted in the urine. A normal reaction can usually be restored in this way without administration of alkali. Recovery can be hastened, however, if sodium bicarbonate is also administered in moderate amounts. This seems advisable as a rule, particularly if it can be guided by repeated determinations of the plasma bicarbonate. Excessive amounts must be avoided, as they may cause a damaging degree of alkalosis. The same principles apply to treatment in the other conditions.

In the *acidosis of renal insufficiency* conditions are complicated by the inability of the kidney to excrete selectively excess of acid or alkali, and to readjust the relative quantities of the electrolytes. The ability to utilize ammonia to neutralize acid radicals is also lost. There is a retention of fixed acids (PO_4, SO_4, possibly others), and a depletion of fixed base. Here special care is required to adjust properly the dose of sodium bicarbonate and sodium chloride administered so as to restore normal proportions as nearly as possible. As the condition is a terminal one any benefit secured can be only temporary and symptomatic. (See also Table 74.)

Differentiation of a primary alkali deficit from a primary CO_2 deficit, both of which show a reduction in the alkali reserve, can usually be made on clinical grounds alone. If this cannot be done, and if active treatment is needed, determination of the pH or CO_2 tension is important, as the treatment indicated in the two conditions is entirely different.

Clinical Methods

Whenever a disturbance of the acid-base equilibrium is suspected a determination of the CO_2 combining power, or the CO_2 content of the plasma, should be carried out if facilities are available. If they are not, helpful information may still be obtained from the simpler procedures which are next described. Table 74, taken from Van Slyke, shows the relative significance of these indirect tests, and gives the values which may be expected normally, and in acidosis of varying severity.

Marked *ketonuria* in cases of starvation, inadequate carbohydrate intake, or diabetes mellitus, indicates the presence of an acidosis, but does not give an accurate estimate of its severity.

If renal function is normal, a high ammonia N/urea N ratio, and particularly an in-

crease in the *sum of titratable acid + ammonia* in the urine is indicative of acidosis, and a rough measure of its severity in disturbances of the primary alkali deficit type. The CO_2 combining capacity of the plasma can be estimated roughly from it by the formula of Fitz and Van Slyke:

$$CO_2 \text{ capacity} = 80 - 5\sqrt{\frac{D}{W}}$$

D = titratable acid + ammonia (in ml. of N/10 acid) excreted in 24 hours.
W = body weight in kilograms.

Tolerance for Bicarbonate (Sellards). This is a simple procedure of considerable practical value as a test for acidosis when more precise methods are not available. Give by mouth (or intravenously if necessary) 5 Gm. sodium bicarbonate in 100 ml. of water. After one-half hour secure a specimen of urine and test the reaction with litmus. If still acid, give an additional 5 Gm. of bicarbonate, and repeat until the urine is alkaline, boiling the specimen if the reaction is doubtful. In normal individuals 10 Gm. usually produces an alkaline urine, and 0.5 Gm. per kilogram always does so. In acidosis larger amounts are required. A negative result (indicating absence of acidosis) is usually reliable. A positive result is not dependable in renal insufficiency, or other conditions in which the electrolytes are depleted. In these conditions caution is necessary, as an alkalosis may be produced by overdosage before the urine becomes alkaline.

Alveolar CO_2 Tension. The alveolar CO_2 tension, as determined by Marriott's method, approximates that in venous blood, which is normally about 45 mm. of Hg, about 5 mm. higher than in arterial blood. It is of value in that from it the approximate bicarbonate content of the plasma usually can be inferred. In a thin-walled rubber bag of 1500-ml. capacity (like the inner bag of a football) put 1 liter of air (600 ml. for infants), and clamp the exit tube. If the subject is coöperative, close the nose with a clip, and insert a mouth piece. At the end of a quiet expiration connect the mouth piece with the bag. The patient rebreathes the air in the bag for 25 seconds, taking five inspirations moderately deeper than the average, during this period. After the fifth expiration clamp the bag and replace the mouth piece with a glass tube drawn out to a capillary tip. Insert the tip in a small test tube in which has been put a few ml. of 0.01 N sodium hydroxide solution containing 0.002 per cent phenol red as indicator. Release clamp and press the bag, forcing a current of air through the solution until the color becomes constant. Compare at once with the color of the standard tubes, which must be of the same diameter. These contain M/15 solutions of monobasic and dibasic phosphate (with phenol red in the same concentration) in proportions corresponding to fixed CO_2 tensions, from 10, 15, . . . to 45 mm. of Hg. These can be purchased, or prepared according to directions found in special texts. In sealed tubes they keep for several months. If the subject cannot coöperate, for the mouth piece substitute a close-fitting rubber mask which is held tightly to the face during the rebreathing period. The procedure is not applicable to patients with circulatory failure or with primary respiratory disturbances. In cases of primary alkali deficit it is the best simple substitute for direct determination of the plasma bicarbonate.

CO_2 Combining Power (Capacity) of the Plasma. This may be determined easily by gas analysis. It has about the same significance as the total CO_2 content, although they are not numerically equal, the combining power being usually about 3 vol. % higher.

Equilibration of the Plasma. (1) Without stasis collect venous blood with the apparatus shown on p. 928, or by means of an all-glass syringe containing a few crystals of potassium oxalate, discharging it as soon as possible beneath paraffin oil in a centrifuge tube. Undue loss of CO_2 to the air should be avoided until the plasma is in the apparatus. However, a slight degree of exposure does not affect the result significantly. (2) Centrifuge as soon as possible, and pipet the clear plasma into a separatory funnel, or store in the refrigerator under oil. (3) Attach the funnel (p. 809) to a bottle containing moist

Condition of Subject	(a) *Plasma CO_2 in Vol. %* (b) *Plasma Bicarbonate in mM*	*Corresponding Results of Indirect Tests for Acidosis*					
		24-hour excretion of 0.1 N Acid + Ammonia*		*Carbon Dioxide of Alveolar Air*		*Sodium Bicarbonate Required to Turn Urine Alkaline*	
		(a) *Ml. per Kg.* (b) *Ml. per 60 Kg. Subject*	*Reliability in Diabetes*	(a) *Tension in mm.* (b) *Approximate %*	*Reliability in Diabetes*	(a) *Gm. per Kg.‡* (b) *Approximate Gm. per. 60 Kg. Subject*	*Reliability in Diabetes*
Normal resting adult; extreme limits	(a) 73–55 (b) 31–23	(a) 0–27 (b) 0–1600	Good	(a) 53–35 mm. (b) 6.8–4.7%	May indicate some acidosis in its absence	(a) 0–0.5 (b) 0–30	May indicate acidosis in its absence
Mild acidosis. No pronounced symptoms	(a) 55–40 (b) 23–17	(a) 27–65 (b) 1600–4000	Good	(a) 35–27 mm. (b) 4.7–3.6%	May indicate more acidosis than is present	(a) 0.5–0.8 (b) 30–50	May indicate much more acidosis than is present
Moderate to severe acidosis. Symptoms may be apparent	(a) 40–30 (b) 17–12	(a) 65–100 (b) 4000–6000	Liable† to considerable error in either direction	(a) 27–20 mm. (b) 3.6–2.7%	Good	(a) 0.8–1.1 (b) 50–65	May indicate much more acidosis than is present
Severe acidosis. Symptoms of acid intoxication	(a) Below 30 (b) Below 12	(a) Over 100 (b) Over 6000	Liable to considerable error in either direction	(a) Below 20 mm. (b) Below 2.7%	Good	(a) Over 1.1 (b) Over 65	May indicate much more acidosis than is present

In nephritis neither the NH_3+ acid excretion-rate nor the amount of Na_2HCO_3 required to turn the urine alkaline is at all reliable as a measure of alkali deficit.

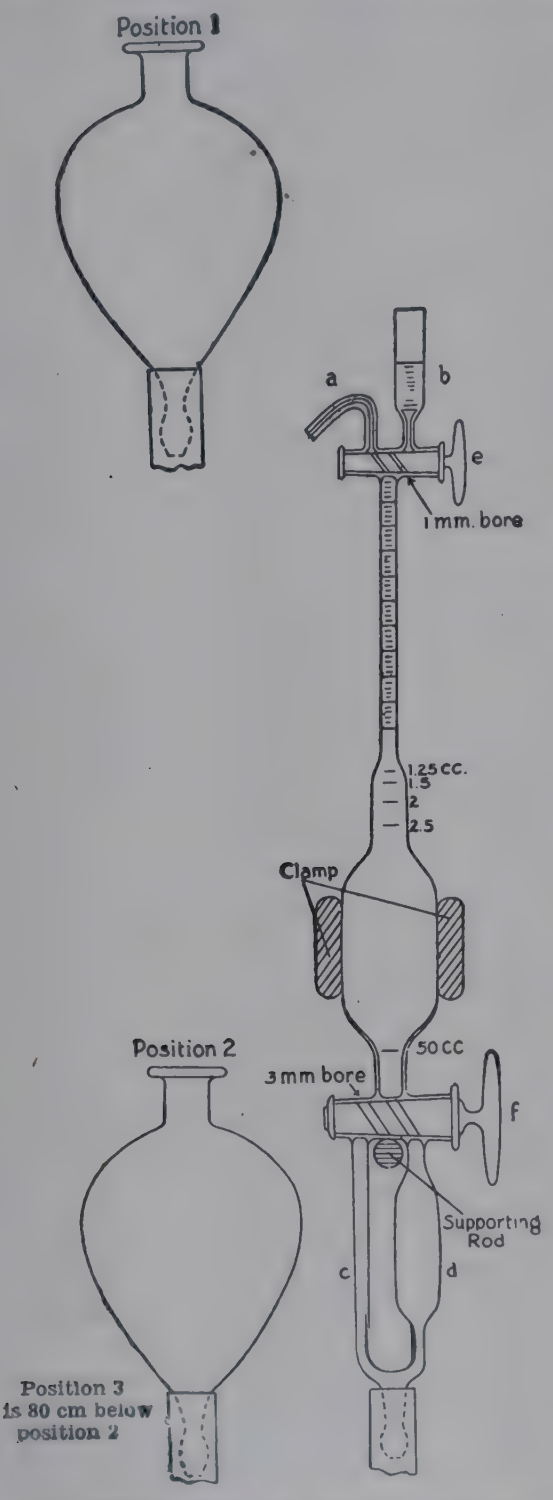
Taken by permission of the authors from Peters and Van Slyke, "Quantitative Clinical Chemistry."

*Measured either in twenty-four-hour urine or from shorter period calculated to twenty-four-hour basis.

†After bicarbonate administration likely to indicate more acidosis than is present.

‡The figures tabulated in this column also indicate the doses of bicarbonate necessary to restore the alkaline reserve to normal from acidosis of the severity indicated by the corresponding plasma CO_2 figures in the first column, according to the results of Palmer and Van Slyke.

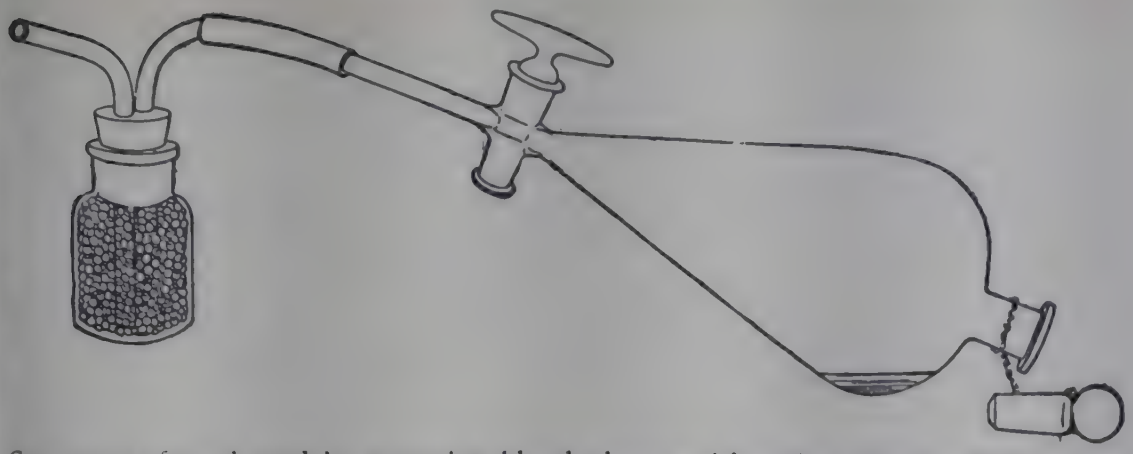
glass beads and after a *normal* inspiration, blow one complete expiration through the apparatus, the current passing from beads to funnel. Close both ends of funnel just before finish. (4) To equilibrate, for two minutes rotate funnel so as to distribute plasma in a thin layer.



Van Slyke's plasma bicarbonate apparatus.

Analysis. (1) Fill the entire apparatus (at left) with mercury, and close stopcock *e*. (2) Make sure that there are no leaks, and particularly that no air seeps through the pressure tubing when the apparatus is exhausted. It is a great advantage to have the improved form of the apparatus with a Shohl trap fused to the lower end to catch such leakage. (3) Put 1 ml. of CO_2-free water in cup *b*. (4) With leveling bulb in "position 2" and cock *f* as shown, slightly open cock *e*, and run 1 ml. of plasma beneath water in cup with the tip of the pipet in the bottom of the cup, so that the plasma flows practically directly into the buret. The pipet should be graduated to deliver between two marks, and not to the tip. (5) Add a drop of octyl alcohol. (6) When capillary above cock *e* contains only the alcohol run 0.5 ml. of a 1 : 10 dilution of concentrated lactic acid into the apparatus, stopping just when the Hg reaches the 2.5-ml. mark. (7) Close cock *e* and seal with a drop of Hg in cup *b*. (8) Place leveling bulb in "position 3," allow Hg to run down to 50-ml. mark. (9) Close cock *f*, remove apparatus from clamp, and mix contents by inverting 15 times. (10) Replace in clamp, open cock *f*, and draw fluid into *d*, leaving capillary of *f* full, and allowing no gas to follow. (11) Reverse *f*, raise leveling bulb, and allow Hg to enter from *c* and rise slowly and *without oscillation*. (12) With Hg in leveling bulb higher than that in the apparatus by one-thirteenth of the height of the water column on the mercury, above the cock *f*, read the gas volume. (13) Calculate volumes % of CO_2 by the use of Table 75, which corrects for temperature, pressure and other factors. (14) Immediately clean apparatus with CO_2-free water, and let stand filled with water.

Total CO_2 Content of Plasma. The total CO_2 content of the plasma is recommended by Van Slyke as preferable to the CO_2 combining power, and should always be done if the pH is to be determined or calculated. It is determined in the same manner except that the plasma is not equilibrated with CO_2, but is pipetted directly into the cup from beneath paraffin oil. Table 76 is used for correcting the gas volume. The greatest care must be taken not to permit any exposure of the blood to air (except the brief period it is exposed in the narrow stem of the pipet). The paraffin oil retards but does not prevent diffusion of gases, and during centrifugation additional precautions must be taken to prevent some loss of CO_2 (replacing the oil with soft paraffin or filling the



Separatory funnel used in saturating blood plasma with carbon dioxide. The bottle contains glass beads. (*J. Biol. Chem.*, 30:289, 1917.)

tube to the brim with oil, and closing with a rubber vaccine-bottle stopper so as to eliminate air bubbles completely). For one not experienced in this technic the CO_2 combining capacity is the safer procedure.

The CO_2 content can also be determined by titration (Van Slyke et al., 1119).

CO_2 Combining Power, or Content, of Whole Blood. The CO_2 combining power, or content, of whole blood may be determined in a similar manner, except that care must be taken that all red cells are washed into the buret, and after the gases have been extracted and the volume read, 1 ml. N sodium hydroxide is drawn into the buret from the cup under slight negative pressure to absorb the CO_2. The volume is then read again. The difference between the readings measures the CO_2. This is corrected in the same manner as for plasma CO_2, except that the subtraction of the third column is omitted.

Arterial blood may be obtained safely in a similar manner. A very sharp hypodermic needle, about No. 20 gauge, with a 45-degree bevel is inserted through the skin at an angle of 45 degrees, and plunged into the radial artery at the point of maximum pulsation. Follow withdrawal of needle by obliterating pressure for two minutes and moderate pressure with a gauze compress for two to three hours. Blood which is practically arterial can be obtained by immersing the forearm in water at 45° C. for 10 minutes, until the veins are widely dilated, and then drawing blood from a vein on the dorsum of the hand near the knuckles.

Oxygen Combining Capacity of Blood. The oxygen combining capacity of the blood, the standard method for determination of hemoglobin, may be measured also with Van Slyke's plasma bicarbonate apparatus. (1) Aerate 3 ml. of CO-free oxalated blood in a separatory funnel by rotating it for a few minutes so that the blood is distributed over the walls in a thin film. (2) Wash the apparatus twice with water. (3) Introduce through the cup 6 ml. water, 0.3 ml. of 1 per cent saponin solution (Merck), and 3 drops of caprylic alcohol. (4) Evacuate, shake thoroughly, and expel the air extracted. Repeat until air is all extracted. (5) Expel solution into cup. (6) Stir blood thoroughly, fill 2-ml. pipet, and (7) with the tip of the pipet in the bottom of the cup, under slight negative pressure, allow blood to enter the buret slowly, along with part of the solution. (8) Add 0.1 ml. of 20 per cent potassium ferricyanide solution (boiled and cooled, to expel air) with the last of the solution. (9) Seal the capillary in cup *b* with a drop of Hg, evacuate the bulb so far that no Hg can be shaken up into the bulb, and shake for three minutes so as to whirl the solution about in the bulb but to avoid violent agitation. (10) Release vacuum, and under slight negative pressure run 1 ml. of gas-free N sodium hydroxide into buret, followed by a drop of Hg. (11) Let stand until alkali has drained down from walls of buret (absorbing the CO_2), draw the solution into the trap below the chamber, and read the gas volume. (12) Multiply this by the correction factor from column 2, Table 76, and subtract 2.1 to correct for N and physically dissolved O. (13) Multiply by

Table 75

TABLE FOR CALCULATION OF CARBON DIOXIDE COMBINING POWER OF PLASMA*

Observed Vol. Gas × Barometer	*Ml. of CO_2 Reduced to 0° C. 760 mm. Bound as Bicarbonate by 100 ml. of Plasma*				*Observed Vol. Gas × Barometer*	*Ml. of CO_2 Reduced to 0° C. 760 mm. Bound as Bicarbonate by 100 ml. of Plasma*			
760	15°	20°	25°	30°	760	15°	20°	25°	30°
0.20	9.1	9.9	10.7	11.8	0.60	47.7	48.1	48.5	48.6
1	10.1	10.9	11.7	12.6	1	48.7	49.0	49.4	49.5
2	11.0	11.8	12.6	13.5	2	49.7	50.0	50.4	50.4
3	12.0	12.8	13.6	14.3	3	50.7	51.0	51.3	51.4
4	13.0	13.7	14.5	15.2	4	51.6	51.9	52.2	52.3
5	13.9	14.7	15.5	16.1	5	52.6	52.8	53.2	53.2
6	14.9	15.7	16.4	17.0	6	53.6	53.8	54.1	54.1
7	15.9	16.6	17.4	18.0	7	54.5	54.8	55.1	55.1
8	16.8	17.6	18.3	18.9	8	55.5	55.7	56.0	56.0
9	17.8	18.5	19.2	19.8	9	56.5	56.7	57.0	56.9
0.30	18.8	19.5	20.2	20.8	0.70	57.4	57.6	57.9	57.9
1	19.7	20.4	21.1	21.7	1	58.4	58.6	58.9	58.8
2	20.7	21.4	22.1	22.6	2	59.4	59.5	59.8	59.7
3	21.7	22.3	23.0	23.5	3	60.3	60.5	60.7	60.6
4	22.6	23.3	24.0	24.5	4	61.3	61.4	61.7	61.6
5	23.6	24.2	24.9	25.4	5	62.3	62.4	62.6	62.5
6	24.6	25.2	25.8	26.3	6	63.2	63.3	63.6	63.4
7	25.5	26.2	26.8	27.3	7	64.2	64.3	64.5	64.3
8	26.5	27.1	27.7	28.2	8	65.2	65.3	65.5	65.3
9	27.5	28.1	28.7	29.1	9	66.1	66.2	66.4	66.2
0.40	28.4	29.0	29.6	30.0	0.80	67.1	67.2	67.3	67.1
1	29.4	30.0	30.5	31.0	1	68.1	68.1	68.3	68.0
2	30.3	30.9	31.5	31.9	2	69.0	69.1	69.2	69.0
3	31.3	31.9	32.4	32.8	3	70.0	70.0	70.2	69.9
4	32.3	32.8	33.4	33.8	4	71.0	71.0	71.1	70.8
5	33.2	33.8	34.3	34.7	5	71.9	72.0	72.1	71.8
6	34.2	34.7	35.3	35.6	6	72.9	72.9	73.0	72.7
7	35.2	35.7	36.2	36.5	7	73.9	73.9	74.0	73.6
8	36.1	36.6	37.2	37.4	8	74.8	74.8	74.9	74.5
9	37.1	37.6	38.1	38.4	9	75.8	75.8	75.8	75.4
0.50	38.1	38.5	39.0	39.3	0.90	76.8	76.7	76.8	76.4
1	39.1	39.5	40.0	40.3	1	77.8	77.7	77.7	77.3
2	40.0	40.4	40.9	41.2	2	78.7	78.6	78.7	78.2
3	41.0	41.4	41.9	42.1	3	79.7	79.6	79.6	79.2
4	42.0	42.4	42.8	43.0	4	80.7	80.5	80.6	80.1
5	42.9	43.3	43.8	43.9	5	81.6	81.5	81.5	81.0
6	43.9	44.3	44.7	44.9	6	82.6	82.5	82.4	82.0
7	44.9	45.3	45.7	45.8	7	83.6	83.4	83.4	82.9
8	45.8	46.2	46.6	46.7	8	84.5	84.4	84.3	83.9
9	46.8	47.1	47.5	47.6	9	85.5	85.3	85.2	84.9
0.60	47.7	48.1	48.5	48.6	1.00	86.5	86.2	86.2	85.7

*The temperature figures at the heads of columns represent the room temperature at which the samples of plasma are saturated with alveolar carbon dioxide and analyzed. It is assumed that both operations are performed at the same temperature. The figures have been so calculated that, regardless of the room temperature at which saturation and analysis are performed, the table gives the volume (reduced to 0° C., 760 mm.) of carbon dioxide that 100 ml. of plasma are capable of binding when saturated at 20° with carbon dioxide at approximately 41 mm. tension. It then figures in the table are multiplied by 0.94 they give, within 1 or 2 per cent, the carbon dioxide bound at 37° C.

Table 76

TABLE FOR OTHER BLOOD GAS DETERMINATIONS

Room Temperature, °C.	*For Oxygen Determinations*	*For Total CO_2 Determinations*	
	Correction for Reduction to 0°C., 760 mm.	*Correction for Dissolved Air*	*Correction for Reduction to 0°C., 760 mm.*
15	$46.6 \times \frac{\text{Barometer}}{760}$	0.052	$100.2 \times \frac{\text{Barometer}}{760}$
16	$46.4 \times \frac{\text{Barometer}}{760}$	0.051	$99.5 \times \frac{\text{Barometer}}{760}$
17	$46.2 \times \frac{\text{Barometer}}{760}$	0.050	$98.9 \times \frac{\text{Barometer}}{760}$
18	$45.95 \times \frac{\text{Barometer}}{760}$	0.049	$98.3 \times \frac{\text{Barometer}}{760}$
19	$45.75 \times \frac{\text{Barometer}}{760}$	0.048	$97.8 \times \frac{\text{Barometer}}{760}$
20	$45.5 \times \frac{\text{Barometer}}{760}$	0.047	$97.2 \times \frac{\text{Barometer}}{760}$
21	$45.3 \times \frac{\text{Barometer}}{760}$	0.046	$96.6 \times \frac{\text{Barometer}}{760}$
22	$45.05 \times \frac{\text{Barometer}}{760}$	0.045	$96.0 \times \frac{\text{Barometer}}{760}$
23	$44.85 \times \frac{\text{Barometer}}{760}$	0.045	$95.4 \times \frac{\text{Barometer}}{760}$
24	$44.6 \times \frac{\text{Barometer}}{760}$	0.044	$94.8 \times \frac{\text{Barometer}}{760}$
25	$44.4 \times \frac{\text{Barometer}}{760}$	0.043	$94.2 \times \frac{\text{Barometer}}{760}$
26	$44.15 \times \frac{\text{Barometer}}{760}$	0.042	$93.6 \times \frac{\text{Barometer}}{760}$
27	$43.9 \times \frac{\text{Barometer}}{760}$	0.041	$93.1 \times \frac{\text{Barometer}}{760}$
28	$43.65 \times \frac{\text{Barometer}}{760}$	0.040	$92.4 \times \frac{\text{Barometer}}{760}$
29	$43.4 \times \frac{\text{Barometer}}{760}$	0.040	$91.8 \times \frac{\text{Barometer}}{760}$
30	$43.15 \times \frac{\text{Barometer}}{760}$	0.039	$91.2 \times \frac{\text{Barometer}}{760}$

0.746 to get hemoglobin in Gm. per 100 ml., or by 5.41 to get per cent of hemoglobin in terms of Haldane's standard (18.5 vol. %). Studies in this country indicate that the normal O combining capacity of young adult males is 20.9 vol. %, corresponding to 15.6 Gm. per 100 ml., according to the usual factor, 1 Gm. hemoglobin = 1.34 ml. O combining capacity. To get percentage of hemoglobin according to this standard, multiply O combining capacity by 4.78.

Oxygen Content of Blood. The oxygen content of either arterial or venous blood can be determined, with the following slight changes in procedure, but the blood must be protected from any exposure to the air, as above described. The saponin and ferricyanide are conveniently combined in a single solution: Dissolve 6 Gm. potassium ferricyanide and 3 Gm. saponin in 1 liter water. At step (3) introduce into the buret 10 ml. of this solution with 3 drops of caprylic alcohol, and (4) extract the air. (5) Expel 6 ml. of the solution into the cup, retaining 4 ml. in the buret. (7) Introduce the blood, washing in with 1 ml. of solution. The remaining 5 ml. are discarded. Step (8) is omitted. (12) After multiplying the observed volume by the correction factor in the second column of the table subtract 1.36 vol. % to correct for N. To get O bound by hemoglobin, subtract instead 1.5 vol. % for venous blood, and 1.7 vol. % for arterial blood.

The per cent of *O saturation* is determined by dividing the observed O content by the O combining capacity. The O unsaturation (in vol. %) is determined by subtracting the O content from the O capacity. Normal arterial blood is 95 per cent saturated.

These analyses can be done more easily and more precisely with the manometric apparatus of Van Slyke, but it is considerably more expensive.

Tests for Blood

Color Tests. Chemical tests for occult blood in the feces, urine, etc. are described elsewhere (pp. 836 and 856). As these depend upon oxidation reactions, although very sensitive they are not specific and are reliable only when interfering substances can be excluded.



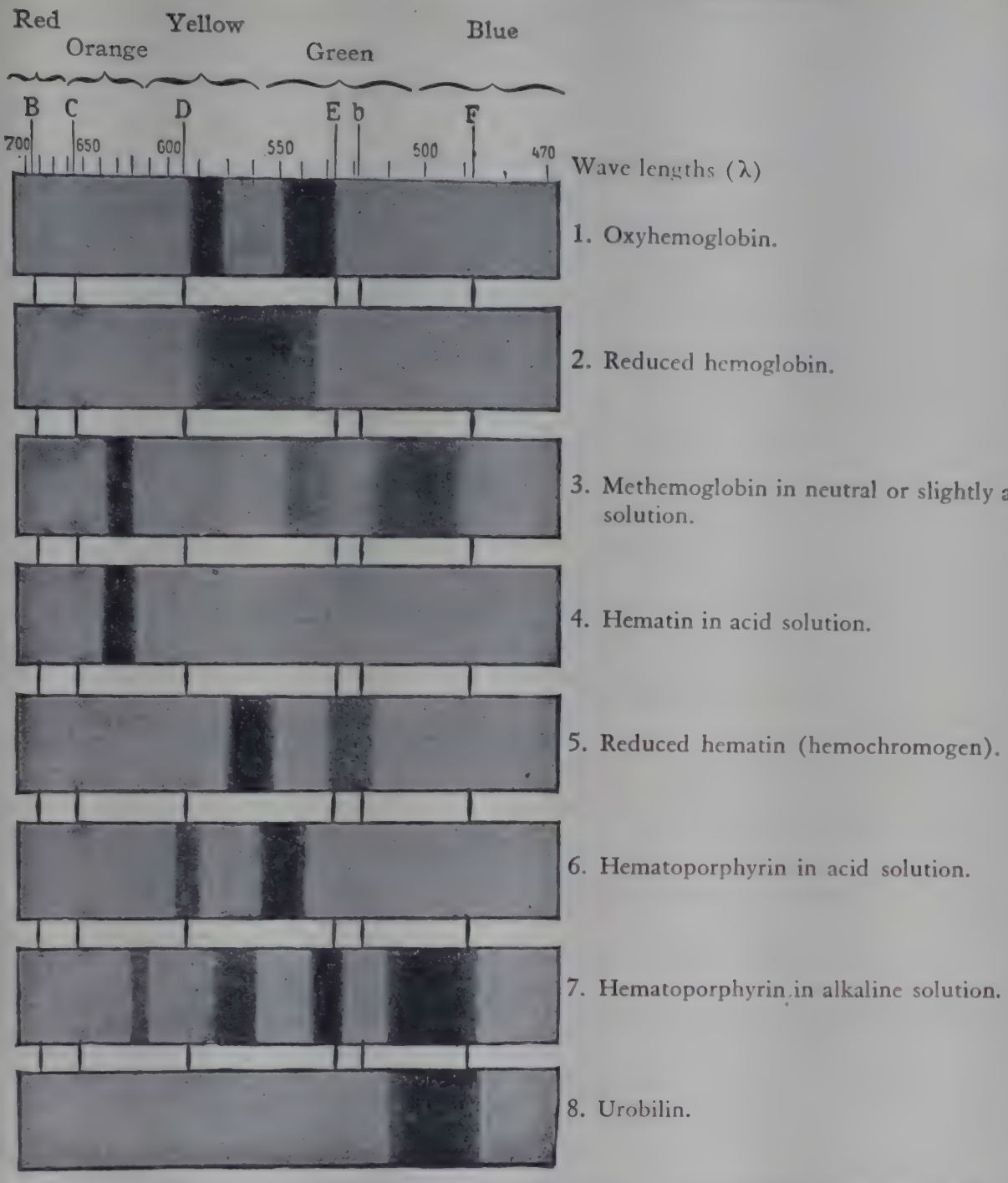
Teichmann's hemin crystals. (Todd, after Jakob.)

Hemin Crystal Test (Teichmann). This test is not sensitive, but a positive result is conclusive evidence of the presence of blood pigments. Prepare a solution (stable) of 0.1 Gm. each of potassium iodide, potassium bromide, and potassium chloride in 100 ml. glacial acetic acid. Mix a few drops with some of the material on a slide, apply a coverglass, and *gently* warm until bubbles begin to appear. Then cool *slowly,* and examine for the characteristic dark-brown crystals.

Hemochromogen Crystals (Donogány). Mix 1 drop each of suspected fluid, pyridine, and 20 per cent sodium hydroxide on slide, and let dry. If blood is present, radiating needles will form after several hours.

Spectroscopic Tests. Spectroscopic tests for blood pigment are conclusive if reactions are definitely positive, but they require a relatively high concentration of pigment. The degree of concentration influences their appearance, and one should start with a relatively concentrated solution, diluting cautiously until the bands are typical. The small direct-vision (hand) spectroscope usually suffices, but for such procedures as detecting and differentiating between methemoglobin and sulfhemoglobin in clinical conditions a more powerful instrument is often required. A wave-length scale is a convenient attachment. Daylight or strong artificial light (such as the "daylite" lamp) is used. The solution is placed in a small test tube or, preferably, a flat cell with a thickness of about 1 cm. Before use, Frauenhofer's lines must be brought sharply into focus. The solutions must be clear; they should be filtered or centrifuged if necessary.

Reducing agents, such as ammonium sulfide or Stokes' solution, are employed. These



The most important clinical spectra. (Modified from Monographic Medicine, New York, D. Appleton & Co.)

must be fresh, and, before use, the sulfide must be warmed to about 50° C. To prepare Stokes' solution, dissolve 3 Gm. $FeSO_4$ in cold water, add cold aqueous solution of 2 Gm. tartaric acid, and make up to 100 ml. Immediately before use, add strong ammonium hydroxide until precipitate first formed is dissolved.

Material that is uncontaminated, relatively fresh, and in relatively concentrated aqueous solution may give any or all of the upper three spectra, a few drops of reducer changing the first or third to the second.

For old dried blood stains the following procedures may be tried:

1. The suspected stain is dissolved in 1 to 2 ml. of 10 per cent sodium hydroxide, heated almost to boiling, cooled, and a few drops of reducer added. Examination shows Spectrum 5.

2. Old and relatively uncontaminated stains are dissolved in potassium cyanide solution and warmed to 40° C. Examination shows cyanhematin (poorly defined bands resembling Spectrum 2). Reduction with ammonium sulfide gives cyanhemochromogen

(similar to Spectrum 5). In cyanide poisoning, blood pigment in walls or lumen of stomach shows cyanhematin.

3. With very old and relatively uncontaminated material, if insoluble in potassium cyanide solution, a small fragment is mixed with a few drops concentrated sulfuric acid, and crushed and rubbed between two glass slides. Examination shows Spectrum 6.

4. If very old and much mixed with other material, the stain is pulverized, mixed with a few ml. concentrated sulfuric acid, and allowed to stand 24 hours. The mixture is filtered through glass wool or asbestos, and the filtrate poured into 10 times its volume of distilled water. The brown precipitate is washed several times, and dried. It is then dissolved in a mixture of equal parts of absolute alcohol and strong ammonium hydroxide, and filtered. Examination shows Spectrum 7, which, upon acidification, will change to a faint Spectrum 6.

It is better, however, especially with much contamination, to prepare an ethereal, acid extract. This not only frees the dried blood from contaminating substances but makes possible a concentration of the pigment. After the material is ground thoroughly with water, if it is not already in liquid form, it is mixed gently with an equal volume of neutral ether. The ether extract is rejected, and to 10 ml. of residue are added 3 to 5 ml. glacial acetic acid. This is shaken thoroughly with an equal volume of ether. If the ether does not separate readily, it is mixed gently with a few drops of alcohol. The ethereal extract is removed, and evaporated to a small bulk for use in tests. Examination will show spectrum of acid hematin, which, however, in ethereal solution, resembles Spectrum 3 more than 4. It is made alkaline with strong ammonium hydroxide, cooled, mixed well, and allowed to separate; a few drops of reducer are then added to the ammoniacal extract. Examination shows Spectrum 5.

Donogány's Method. Donogány's method increases the delicacy of the spectroscopic test, and is also a color test. The pigment is dissolved with 20 per cent sodium hydroxide, fresh pyridine is added and, if necessary, fresh ammonium sulfide. The mixture is filtered. The filtrate will be more or less orange-red according to blood content, and will show Spectrum 5.

The spectrum of *carbon monoxide hemoglobin* resembles that of oxyhemoglobin except that the bands are shifted slightly toward the violet, and are closer together. Addition of reducing agents does not change it to reduced hemoglobin (demonstrable only in concentrations of at least 30 per cent saturation). Chemical tests are more sensitive (see below). To detect and measure small quantities gasometric determinations are necessary.

Demonstration of *methemoglobin* is important in poisoning with certain drugs such as acetanilid. *Sulfhemoglobin,* long confused with the preceding and often present with it, has a similar band which persists after the addition of reducing agents. It is abundant in cases of "enterogenous cyanosis." The spectrum of *acid hematin* which also has a similar band near C is changed by reducing agents to Spectrum 5.

Color Tests for Carbon Monoxide in Blood

Katayama's Test. Put 5 drops of suspected blood and 5 drops of normal blood in each of two test tubes. Dilute each to 10 ml. with water, add 5 drops of fresh yellow ammonium sulfide solution, and acidify slightly with acetic acid. Normal blood turns a dirty greenish-brown color; blood containing carbon monoxide retains more or less of a reddish tint.

Haldane's Dilution Test. Dilute 1 ml. of suspected blood to 100 ml. with water, and similarly 1 ml. of normal blood as a control. Compare the colors against a white background. Blood containing CO has a purplish pink tint as compared with the yellowish red of normal blood.

Sayers and Yant's Tannic Acid Method. This gives an approximate quantitative estimate of the CO present. In a small test tube dilute 0.1 ml. suspected blood with 0.4 ml.

dilute ammonia (1 ml. strong ammonia per liter). Mix and add 1.5 ml. freshly prepared 1 per cent tannic acid solution. Let stand overnight and compare the color either with permanent standards or standards prepared simultaneously as follows: Bubble CO or illuminating gas through 1 ml. oxalated blood until saturated. Dilute this and also 1 ml. normal blood with 4 ml. dilute ammonia. Make up a series of mixtures of these laked bloods (in 0.5 ml. volume) corresponding to 10, 20, 30, 40, 50, and 60 per cent saturation. Add 1.5 ml. tannic acid solution to each.

In none of these tests are the color differences well marked if the CO saturation is under 20 per cent. In acute poisoning the saturation usually ranges from 25 per cent to 60 per cent or more, but it falls quickly after the patient is removed from the source of poisoning (30 to 50 per cent per hour in air, about four times as fast in 95 per cent O and 5 per cent CO_2).

CHAPTER 37

Blood Cultures

The demonstration of bacteria in the blood is of great importance in the diagnosis and prognosis of various infections. Only very rarely are the ordinary bacteria sufficiently numerous in the blood to be seen in smears, but it is usually a simple procedure to obtain them by culture.

A transient bacteremia occurs frequently in many of the acute infectious diseases, especially at the onset. It is especially common in the typhoid group of infections, pneumonia, undulant fever, cerebrospinal meningitis, and may occur in other infections. In these cases the presence of a few organisms in the blood is of great diagnostic importance, but is without special significance so far as prognosis is concerned. On the other hand, if they are present in large numbers, especially if they persist, and their number increases, the prognosis becomes grave unless effective specific therapy is available.

From a focal infection anywhere in the body organisms of many kinds may invade the blood stream. As a rule the bactericidal power of the blood is sufficiently great to kill these bacteria, so that the blood remains sterile. Occasionally, however, owing to a diminished resistance or to the high virulence of the infecting organism, the bacteria survive, and appear in the blood in large and increasing numbers. This increase is due, not to multiplication in the blood stream, but to greater invasion from the focus. Such a condition is known as septicemia or sepsis. If an individual lives long enough, secondary suppurating foci may occur, and the condition is termed pyemia. Bacteria found in the blood in these conditions have a much more serious prognostic significance.

Certain organisms of relatively low virulence may grow and multiply on the heart valves, and some escape more or less constantly into the circulation. There may be periods in the chronic cases in which blood cultures are sterile, and repeated tests may be necessary to demonstrate them. Such an endocarditis is most often due to the *Streptococcus viridans*. Hemolytic streptococci cause a rapidly fatal, ulcerative endocarditis.

In the interpretation of the results of blood culture the clinical picture must be considered. The significance of pneumococci in a blood culture is not the same in an early case of lobar pneumonia, in a chronic endocarditis, and in an acute mastoid infection, for example.

Although blood invasion by the staphylococcus may occur, this organism is regularly present on the skin; and blood cultures yielding a few colonies, especially of *Staphylococcus albus,* should be viewed with suspicion, and the results corroborated before accepting them as significant. Diphtheroid bacilli are also commonly present on the skin, and various bacteria from the air may contaminate a culture. However, if the blood is cultured immediately, and if part of it is planted in solid media (blood agar plates), it is usually easy to recognize a contamination as such, should one occur.

In taking blood for culture it is desirable to select a time when the temperature is high or rising. The methods for making cultures vary in details with the type of organism suspected, but the following serves as a good routine.

Technic. The blood is withdrawn from some prominent vein, preferably the median basilic vein of the arm, by means of a sterile 10- or 20-ml. syringe. If Luer syringes are used they may be sterilized, together with a needle, in large plugged tubes in the hot-air sterilizer, or boiled for from 5 to 10 minutes just before obtaining the blood. Large Keidel tubes containing sodium citrate, alone or combined with glucose broth, are convenient if only an occasional culture is made.

The skin over the vein should be scrubbed thoroughly with soap and water, and painted with tincture of iodine. A tourniquet is applied to distend the vein during the puncture. Ten or 15 ml. of blood are withdrawn, and the tourniquet is removed before drawing out the needle. The iodine is then washed off with alcohol, and the puncture covered with sterile gauze or cotton. The blood is added to a 50-ml. flask containing 5 ml. of 1 per cent sterile sodium citrate solution in physiological saline. After removing the stopper the mouth of the flask should be flamed before introducing the blood. The citrate prevents clotting until the blood can be inoculated into the various media. The culture can be made at the bedside when feasible, but the use of the citrate flasks is generally more convenient. The inoculations should be made as soon as possible. The blood can be transferred to the media with a sterile cotton-plugged 10-ml. pipet, or, with a little experience in judging quantities, poured into the following media.

a. One or 2 ml. in each of 2 or more tubes of melted agar cooled to 42° C. The contents of the tubes are mixed and poured into sterile Petri dishes.

b. One or 2 ml. in 2 or more tubes of dextrose brain broth.

c. Five ml. in a flask containing 100 ml. of glucose broth.

The remainder of the citrated blood may be incubated as a control. The cultures should be examined daily for evidences of growth. Colonies developing on the plates should be counted and subcultured for identification. If growth appears in the broth, transfers should be made and smears examined. After 72 hours the broth is subcultured on a blood agar slant, even though no growth is evident. The original cultures should be kept for at least two weeks, and preferably a month, since some bacteria grow slowly when first isolated.

Special modifications of this method are used for some cultures. If *anaerobic organisms* such as anaerobic streptococci are suspected, part of the culture must be incubated under anaerobic conditions. In large laboratories a McIntosh and Fildes jar can be used, or some modification of the pyrogallic acid and sodium hydroxide method. (See section on anaerobic methods.) Fairly satisfactory anaerobiosis can be obtained by inoculating the blood into deep narrow tubes of glucose agar or glucose broth which have been previously heated to remove oxygen, cooled, and covered with sterile petroleum jelly. Some workers make, as a routine, deep tubes of glucose ascitic fluid agar without petroleum jelly in order to obtain varying degrees of oxygen tension in the culture. (See p. 920.) Anaerobic bacilli associated with wound infections seldom invade the blood stream, although the gas bacillus has been obtained in blood culture.

When *B. abortus* is suspected, 10 to 15 ml. of blood are distributed in varying quantities into small flasks containing 30 ml. of meat infusion broth pH 7.5. Several agar slants are inoculated by flooding their surfaces with blood. These are incubated at 37° C. in an atmosphere with a carbon dioxide tension of 10 per cent. Since the organisms grow slowly, the cultures should be examined and subcultured on glucose agar slants from the fourth to the fourteenth day. Ten per cent carbon dioxide tension may be produced by any of the methods described (see p. 920).

The *tubercle bacillus* rarely occurs in the blood except in miliary tuberculosis. In these cases growth can be obtained by laking the blood with sterile distilled water and planting the centrifuged sediment on suitable solid media. Since growth is slow, the moisture in the medium must be conserved by sealing the tubes with paraffin or petroleum jelly after a few days.

Animal Inoculation. Animal inoculation is less used for obtaining bacteria from the blood than from contaminated material, but it is essential in some cases. In *tularemia* the organisms have been obtained from the blood in the first week of the disease by inoculating rabbits or guinea pigs intraperitoneally with from 2 to 4 ml.

Anthrax and *plague* bacilli can be demonstrated by the inoculation of mice or guinea pigs. *Tubercle bacilli* are easily found by guinea-pig inoculation. The *spirochetes* of Weil's disease may be seen in direct smears from the blood, but are difficult to cultivate on artificial media. Their presence, however, can be demonstrated easily by guinea-pig inoculation. When *trypanosomes* cannot be found in smears animals should be inoculated.

In animal inoculation from 1 to 5 ml. of blood are injected intraperitoneally or subcutaneously. After the animal's death, cultures can be made from the heart's blood, liver, and spleen, and smears from these organs usually show the organisms. Characteristic lesions may be found in some cases.

The presence of some of the *rickettsias* and *filtrable viruses* in human blood has been demonstrated by the production of lesions in animals, and by the development of an infection from which, after recovery, they can be proved to be immune. This method is of great use in the diagnosis as well as in the investigation of diseases due to filtrable viruses.

Animal Parasites Found in Blood. The examination of the blood for the parasites of malaria, filariases, kala-azar, and spirochetal fevers has been discussed under their respective headings.

With trypanosomes from human trypanosomiasis, smears from gland juice or cerebrospinal fluid seem more satisfactory to examine than blood smears unless the blood is taken in 5- to 10-ml. quantities and centrifuged in sodium citrate-salt solution.

A method in the diagnosis of trichinosis is to take 5 to 10 ml. of blood from a vein at the time of the migration of the embryos to the muscles (10 to 20 days). The blood is forced out into a centrifuge tube containing 3 volumes of 3 per cent acetic acid, and the sediment examined for trichina larvae.

CHAPTER 38

Examination of the Urine

For routine purposes it usually suffices to examine two single voidings: one, obtained about two hours after the principal meal, which is most likely to show sugar and albumin, if present; and a second, obtained on rising in the morning, which gives a check on the concentrating power of the kidney. A note should always be made regarding the color, turbidity, specific gravity, reaction, microscopic examination of the sediment, and chemical tests for albumin, sugar, and acetone. For quantitative chemical studies it is necessary to collect the total urine for 24 hours, keeping it on ice, or using a preservative (preferably 2 per cent toluol) to prevent bacterial decomposition; or for shorter fixed periods, under standard conditions.

Color. Normally urine varies from pale straw to deep amber, according to its concentration, owing to the pigment urochrome. Rarely, it is pinkish (uroerythrin). In jaundice (bilirubin) it shows varying tints from orange-yellow to deep brown, and a yellowish foam when shaken. Urobilin in large amount also gives it a brownish tinge, but a colorless foam. In hemoglobinuria it shows a clear reddish color. In hematuria it varies from a smoky tinge to opaque bright red or reddish brown. A black tinge suggests melanin, which may be found in melanotic sarcoma, or in the rare constitutional anomaly, alkaptonuria. Unusual tints (of yellow, brown, red, green, or blue) may follow the administration of a great variety of drugs.

Turbidity. Gross turbidity in an old specimen of urine is usually due to the growth of contaminating bacteria, to amorphous urates (if acid), or to carbonates and phosphates (if alkaline). In freshly voided acid specimens it is usually due to infection (pus cells and bacteria), but in alkaline specimens (shortly after a meal) it is often due to carbonates and phosphates which dissolve after the addition of a few drops of dilute acetic acid. A faint opalescence may be due to bacteriuria (without pus cells), as is frequent in typhoid fever. In *chyluria* the urine is opaque and milky white. It follows rupture of a lymph vessel into the urinary passages. It is usually (but not always) due to filariasis. The embryos may be present in the urine.

Specific Gravity. This is significant only when the specimen is collected under standard conditions. If no fluid is taken during the evening and the bladder is emptied on retiring, the early morning voiding should have a specific gravity of 1.020 or higher. A very low specific gravity is usually due to excessive fluid intake, and should be viewed with suspicion in the case of a candidate appearing for physical examination, since pathologic constituents often escape detection in the resulting dilution. (See tests for renal function.) A low specific gravity in the 24-hour specimen may be due simply to a restricted diet, a low salt intake, or copious water drinking.

Reaction. Litmus paper, which is commonly used, usually suffices, but it is a poor indicator. Nitrazine paper is preferable, and with it an approximate determination of the pH can be quickly made provided the specimen is fresh. Normally the reaction is acid except shortly after a meal, when it may be alkaline ("alkaline tide"). In special cases it may be desirable to determine the pH more precisely or to measure the titratable acidity (see p. 840).

Urinary Sediments. For the examination of organized sediments the specimen should be as fresh as possible. If it is not to be examined immediately, it should be kept cold,

or a little formalin should be added. Red cells and casts disintegrate rapidly in warm specimens, particularly if dilute and alkaline.

Sediment is best concentrated by centrifuging 15 ml. of urine at 1500 revolutions per minute for five minutes (not longer), decanting the supernatant urine, and suspending the sediment in the few drops of urine that flow back from the walls of the tube. Take up some of the sediment with a capillary pipet and mount on a slide 1 drop of such size that when covered with a 22-mm. square coverslip it does not flood out from under the coverslip. Examine with dim illumination under the low-power objective, particularly for casts, and identify individual cells present with the high dry objective. A drop of Gram's iodine solution may be added to stain the cells.

In order to secure a uniform degree of concentration, it is customary in the laboratories of the U. S. Naval Medical School to resuspend the sediment (from 15 ml.) in exactly 1.5 ml. of urine. This facilitates the quick determination of an excessive number of red cells or pus cells without recourse to elaborate counting procedures. If desired, however, a sample of the resuspended sediment can be mounted on a hemacytometer and the average number of cells or casts in a 1-sq. mm. field can be determined. This is equal to the number present in 1 cu. mm. of original urine, and from this the total output can be calculated.

Epithelial Cells. Moderate numbers of epithelial cells are constantly present in normal urine. Large cells of the squamous type arise from the bladder or vagina. Smaller cuboidal or spherical cells which are attached to casts or which are filled with coarse refractile granules come from the kidney. It is practically impossible to determine the source of other types of epithelial cells with certainty from their appearance. Sheets of more or less round or caudate epithelial cells are suggestive of pyelitis.

Cells with highly refractile lipoid granules (doubly refractile under a polarizing microscope) are common in the "nephroses."

Renal cells containing *hemosiderin granules* are common in hemochromatosis and occur in the active stage of various hemolytic anemias. *Rous' method* (1918) may be used to demonstrate them. (1) A *freshly* voided specimen of urine is centrifuged and decanted. (2) A little of the sediment is examined for brownish granules (intracellular and extracellular). (3) The rest of the sediment is resuspended in a freshly mixed solution containing equal parts of 2 per cent potassium ferrocyanide and 1 per cent hydrochloric acid. (4) After 10 minutes this is centrifuged and the sediment examined for blue granules (high dry objective). A normal urine should be treated in the same way as a control.

Leukocytes. To differentiate a leukocyte from an epithelial cell it is necessary to see the lobes of the nucleus clearly. This is often impossible unless the preparation is stained (as with Gram's iodine solution), or cleared with a drop of dilute acetic acid, which dissolves the cell granules, and brings out the outline of the nucleus distinctly.

A note should be made of the approximate number per high-power field. A convenient method of recording is: $+^1$ (5 to 20 cells); $+^2$ (20 to 50 cells); $+^3$ (50 to ¾ filled); $+^4$ (packed field). If abundant, note should be made of the volume of packed cells in the centrifuge tube. Precise counts are rarely worth while.

Pus cells in a voided specimen of urine from a female may be of no significance, because of the frequent contamination with vaginal discharge. In a catheterized specimen or in male urine, more than an infrequent leukocyte is pathologic (maximum of 3 per high-power field, if concentrated under standard conditions, and not over 1,000,000 in 24 hours when estimated by the Addis method). In acute infections of the urinary passages large numbers of pus cells are present, often in clumps. Pus cells are frequently found in acute and chronic nephritis. A renal origin can be determined only when they are attached to casts.

In males, the use of two sedimentation glasses will differentiate pus from the urethra from pus coming from the bladder or pelvis of the kidney. If the urine in the first glass

alone is turbid, it indicates urethral pus. The presence of pus cells attached to macroscopic shreds of mucus suggests a chronic prostatitis and posterior urethritis, which may, but need not, be gonorrheal. It is impossible to differentiate between cystitis and pyelitis by examination of urine voided, or catheterized from the bladder. Differentiation must be made clinically or by ureteral catheterization.

In every male with pus cells (in any number) in the urinary sediment, one should examine the *prostatic secretion* obtained as described on p. 49. A drop of 10 per cent acetic acid (and a trace of methylene blue) should be mixed with a little secretion, and a drop examined (⅙-inch objective). Normal secretion contains epithelial cells, lecithin droplets, "amyloid bodies," and an occasional pus cell, up to about 6 per high-power field. More than 10 cells per field is pathologic—particularly if some clumps are present.

Erythrocytes. For the demonstration of red cells in urine, fresh specimens are particularly important. This condition (hematuria) must be distinguished sharply from hemoglobinuria, in which dissolved blood pigment (but not intact cells) is present. The red cells may be morphologically intact, or be swollen, and often laked, so that only shadows remain, or shrunken, crenated, and distorted in varying degrees, and occasionally fragmented. They must be distinguished from vegetable spores, yeast cells, refractile droplets, and certain crystals, which sometimes resemble them. Their usually uniform diameter of 6 to 8μ suffices. In case of doubt, a test for occult blood should be applied to the sediment, but it must be remembered that red cells can be demonstrated microscopically with ease in urine which contains too little blood to give a positive reaction to the benzidine test.

The approximate number of cells per high-power field should be recorded, and, if grossly bloody, the volume of packed sediment: $+^1$, 3–8 cells; $+^2$, 8–30 cells; $+^3$, ¾ filled field; $+^4$, packed field.

Addis Counts. A few red cells are present in the urine of many normal individuals. Addis (1926) kept subjects on a dry diet for a day, allowing the usual foods but no liquids. He then collected the urine for 12 hours (8 P.M. to 8 A.M.) during which fluids were still withheld, concentrated the sediment from 12.5 ml., and resuspended it in 0.5 ml. of urine. In such concentrated acid urine the structures are well preserved. He made *enumeration of the red cells,* etc., by use of a hemacytometer, and calculated the total excretion for the 12-hour period. He found red cells in 40 of 64 normal medical students, the total excretion ranging from 0 to 425,000 cells (820 per ml.). Counts of well over 1,000,000 he regards as pathologic, and in the absence of other sources of bleeding, as evidence of active nephritis. The procedure is too time-consuming to be practicable for routine use but appears to be valuable in special cases in the detection of mild or latent nephritis. (Hematuria occurs only intermittently in these cases.) Adequate information can usually be obtained by examining, in the ordinary way, a fresh preparation of the sediment collected by Addis' method.

In women, contamination with vaginal and even rectal bleeding must be excluded.

Hematuria. Hematuria is most often met with in the following conditions: (1) Trauma. (2) Acute infections of any part of the urinary passages, including renal tuberculosis. (3) Benign or malignant growths, particularly hypernephroma and vesical polyps. (4) Benign prostatic hypertrophy. (5) Renal, ureteral, or vesical calculi. (6) Kinks or strictures of the ureter. (7) Acute nephritis or acute exacerbations of a chronic nephritis, occasionally in arteriosclerotic nephritis. Blood cells in casts are always from the kidney. (8) Poisoning with certain drugs, such as bichloride of mercury, phenol, turpentine, cantharides, oil of sandalwood, methenamine. (9) Chronic passive congestion. (10) Renal infarctions, and bacterial emboli in sepsis. (11) Severe infections, such as smallpox, plague, yellow fever. (12) Certain tropical parasitic infections, as bilharziasis and filariasis. (13) General systemic diseases with a tendency to bleeding, such as purpura and leukemia. (14) In some cases ("idiopathic hematuria") in which no cause can be found.

Hemoglobinuria. Hemoglobinuria occurs most frequently in paroxysmal hemoglobinuria, blackwater fever, and after reactions to transfusions of incompatible blood.

Mucus. Mucus is secreted in the bladder and urinary passages in the form of microscopic threads often combined into ribbon-shaped masses which at times may superficially resemble casts. They are flat, not cylindrical, usually show longitudinal striation, are variable and irregular in size and shape, and taper at the ends which are often split or curled. In the presence of irritation or inflammation of the passages, they may form large masses which mechanically conceal other structures which may be present.

Casts. Casts are formed by coagulation of albuminous material in the lumina of the tubules of the kidney. Their shape and size correspond to that of the tubules in which they are formed. They should be searched for with the ⅔-inch objective, with the light well dimmed. They are cylindrical, fairly uniform in diameter, and have rounded ends.

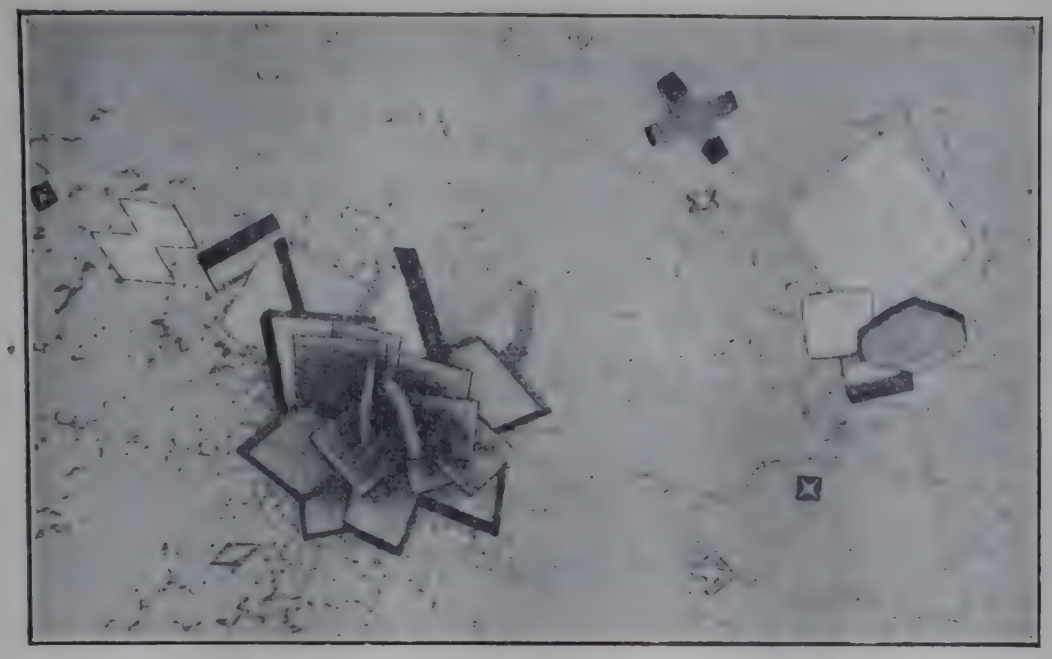


Fatty and waxy casts. (*a*) Fatty casts. (*b*) Waxy casts. (Greene.)

Hyaline casts show no internal structure. *Finely granular casts* are similar, but show fine granulation. Both will be overlooked with bright illumination. They indicate some irritation or inflammation of the kidney. In general, they occur in conditions associated with albuminuria, and have about the same significance (see p. 828). *Cylindroids* are similar to hyaline casts except that their ends are elongated and tapering. They have the same significance. They must be distinguished sharply from broad, flat, strap-shaped strands of mucus, which are not renal in origin. Hyaline and finely granular casts are practically always present if casts of other types are present, and usually greatly outnumber the others. An approximate estimate of the number should be made: if sparse, the number in an entire coverslip preparation; if more numerous, the number per low-power field: ($+^1$, 1 to 5; $+^2$, 5 to 10; $+^3$, 10 to 30; $+^4$, packed). The number of other types of casts, if present, should also be noted separately.

A few hyaline and finely granular casts can be found in many normal individuals. Addis found them in 45 of 74 normal students. The number excreted in a 12-hour period ranged from 0 to 4270 (80 per ml.).

Waxy casts are similar to hyaline casts in showing little or no internal structure, but are more highly refractile, denser, more opaque-looking, often show slits or cracks and



Common sediments of acid urine: Uric acid crystals, calcium oxalate crystals, and amorphous urates. (× 150.) (Courtesy, J. C. Todd: Clinical Diagnosis, Philadelphia, W. B. Saunders Co.)

jagged broken ends. Transitions to hyaline casts occur. They occur most often in advanced chronic nephritis and amyloidosis. They may give a positive amyloid reaction.

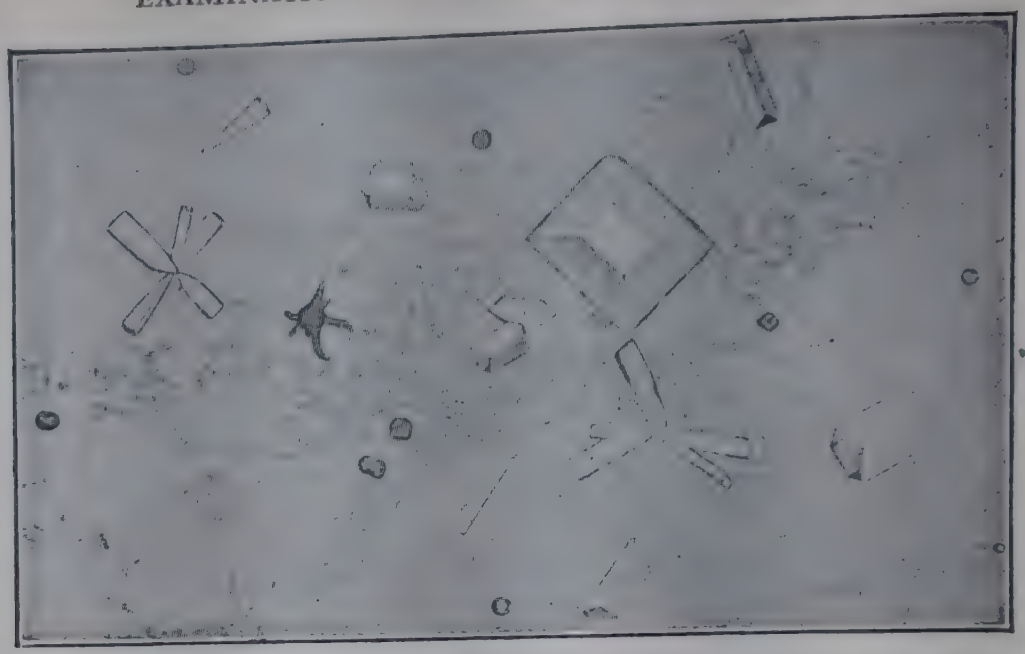
Coarsely granular casts usually indicate nephritis. Renal *epithelial* cells, red cells, or pus cells may be attached to *casts* of any type, or may make up the bulk of the cast. They indicate an active nephritis and usually an acute process. The epithelial cells often show granular degeneration, and the coarsely granular and *fatty casts* contain the products of cellular degeneration. Very broad casts (*renal-failure casts*) are characteristic of the advanced stage of renal disease.

Coarsely granular casts may be simulated by incrustations of precipitate about thick strands of mucus in decomposing specimens.

Unorganized Sediments. The types and quantity of crystalline precipitate depend mainly upon the reaction and degree of concentration of the urine. An excess of any type of crystal is not indicative of a disturbance of metabolism. With few exceptions they are of no practical significance.

The following are found in *acid urines:* (1) *Sodium and potassium acid urate:* amorphous, yellowish-red granules which dissolve on heating, or on adding alkali. (2) *Uric acid:* yellowish crystals, usually of a whetstone shape, often clustered, rarely in flat plates; soluble in alkali and in strong acids but not on heating. (3) *Calcium oxalate:* small, highly refractile, octahedral crystals ("envelope crystals"), or rarely dumbbell-shaped or spherical. They are increased with a diet rich in oxalates (asparagus, spinach, tomatoes, rhubarb). The presence of clusters of calcium oxalate or uric acid crystals in *freshly voided* urine suggests that conditions in the urinary passages are favorable for calculus formation, but does not prove their presence. (4) Rarely, cholesterol, cystin, tyrosin, leucin, xanthin, hematoidin, biliverdin, indigo, melanin, creatinine, hippuric acid, sodium biurate, calcium sulfate, neutral calcium phosphate.

In *alkaline urine* one finds: (1) *Triple phosphate* ($NH_4MgPO_4.6H_2O$) crystals, in coffin-lid or fernlike form, easily soluble in acetic acid. (2) *Ammonium biurate* crystals, yellow, thorn-apple in shape, soluble in acetic acid. (3) *Calcium phosphate*, in slender radiating crystals or flat plates. (4) *Magnesium phosphate* (rarely). (5) *Tricalcium phosphate* [$Ca_3(PO_4)_2$], *Magnesium phosphate* [$Mg_3(PO_4)_2$], and *calcium carbonate* as fine amorphous precipitate, easily soluble in acetic acid, the latter with effervescence. They are the usual cause of turbidity in freshly voided, alkaline normal urine. They are often precipitated by heating an alkaline urine.



Common sediments of alkaline urine: Triple phosphate crystals, calcium phosphate crystals, ammonium urate crystals, and amorphous phosphates. (× 150.) (Courtesy, J. C. Todd: Clinical Diagnosis, Philadelphia, W. B. Saunders Co.)

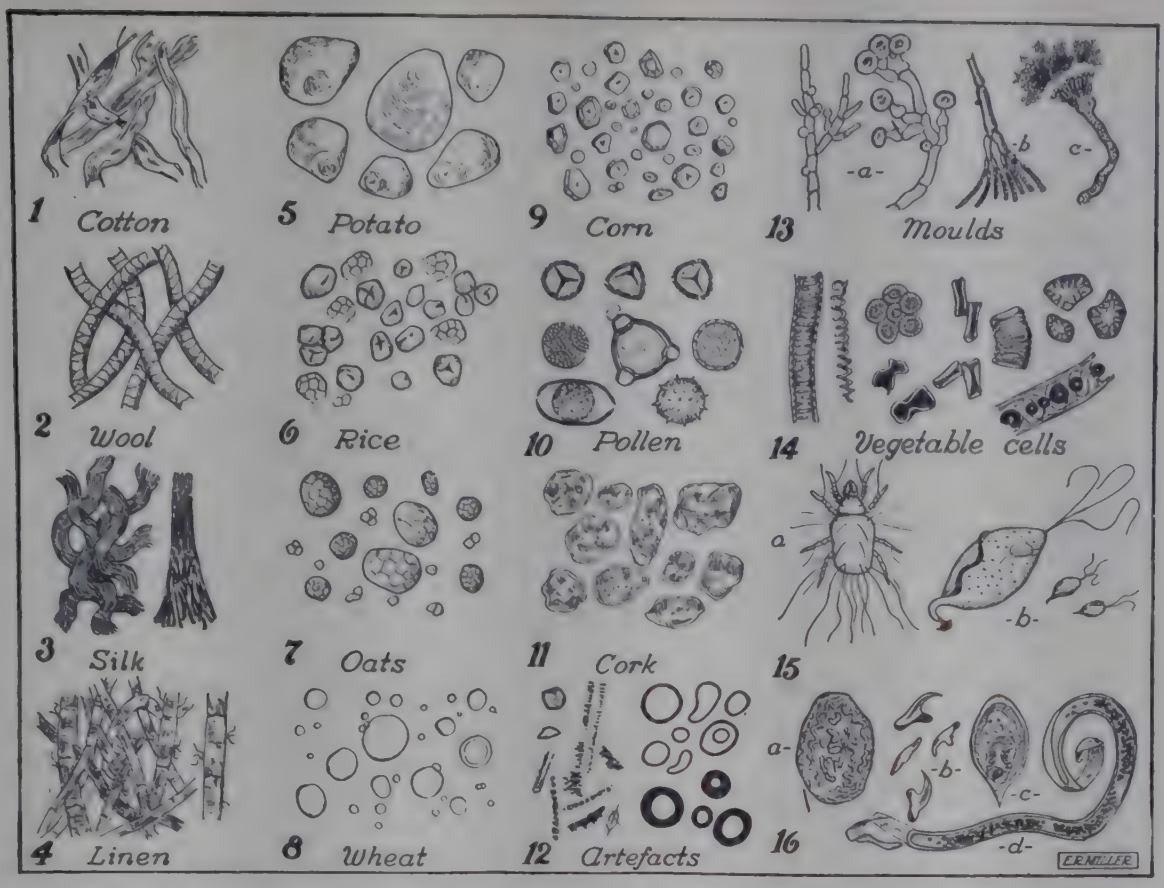
The presence of numerous ammonium biurate and triple phosphate crystals in *freshly* voided urine indicates bacterial decomposition within the bladder. It occurs only where there is stasis, as in urethral obstruction, or paralysis of the bladder musculature, and is usually associated with a secondary acute cystitis.

Cystin crystals occur in the urine (*cystinuria*) in a rare constitutional anomaly which results in an inability of the body to break down protein completely in the normal manner, and which is characterized clinically by a marked tendency to form renal calculi. They appear as transparent, hexagonal plates (sometimes only after concentration of the urine). They can be distinguished from uric acid and other similar crystals by the fact that they dissolve in hydrochloric acid but not in acetic acid; that they dissolve in ammonia, and recrystallize when the ammonia is driven off by gentle boiling; and that when dried and heated they char, and give off an odor of burning wool.

Leucin and *tyrosin* are excreted by patients with marked destruction of liver tissue (acute yellow atrophy), but crystals are rarely found unless the urine is concentrated. Urine should be freed from albumin and filtered (preferably with basic lead acetate, removing excess of lead with hydrogen sulfide). It is concentrated on a water bath to a syrup; urea is removed by several extractions with alcohol; and the residue is extracted with hot, dilute, ammoniacal alcohol. The latter is filtered, concentrated, and allowed to stand. Leucin appears as small, spherical, yellowish crystals which show radial and concentric striations, and which dissolve in hydrochloric acid and boiling glacial acetic acid. Tyrosin appears as yellowish or blackish, fine needles arranged in spherical clusters or sheaves. They can be identified by their solubility in hydrochloric acid and in ammonia (but not in acetic acid), and by the fact that they give a green color when boiled with Mörner's reagent (1 per cent formalin in 55 per cent sulfuric acid).

Sulfonamide Crystals. These may be present in the urine during the administration of any of the sulfa drugs, particularly if the patient is allowed to become dehydrated and the urine volume is scanty. They may be composed of the free drug or of the acetylated form. They cannot be identified with certainty from their structure alone, and if there is doubt, they may be concentrated by centrifugation, suspended in distilled water, and identified chemically by the procedure used in determining sulfonamides in whole blood (see p. 794). The presence of a few crystals is usually of no significance, but large numbers of them constitute a danger signal.

Extraneous Particles. In examining urinary sediments it is important to be familiar



Fibers, starch granules, etc., which may be found in urine sediment. *12* gives appearance under microscope of scratches on old used glass slides. (*15a*) *Tyroglyphus longior,* a mite. (*15b*) *Trichomonas vaginalis.* (*16a*) Egg of *Dioctophyme.* (*16b*) *Echinococcus* hooklets. (*16c*) *Schistosoma* egg. (*16d*) *Wuchereria bancrofti* embryo.

with the various textile fibers and starch grains which are so frequently present, the fibers coming from the clothing and the starch grains from dusting powders. Wool-fiber fragments show bark or scalelike imbrications and are round. Cotton fibers are flattened and twisted, whereas linen ones show a striated flattened fiber with frayed segments as of a cane stalk. Silk shows a glasslike tube with mashed-in ends.

Corn and rice grains are the most common of the starch grains and their nature is immediately disclosed by their blue color when mounted in iodine.

Analysis of Urinary Calculi

(Klopstock and Kowarski.)

There are four principal types of urinary calculi: (1) Uric acid or urates. Acid sodium urate is more common than ammonium urate. (2) Phosphates of calcium and magnesium and calcium carbonate. (3) Calcium oxalate. (4) Cystin and xanthin (rare). (5) Mixed calculi with layers of different composition.

To some extent calculi may be differentiated by their color, the character of the surface and their consistence.

Color. Urate stones are yellow to dark-brownish-red; phosphate, whitish, gray or grayish-yellow; oxalate, chiefly brownish-red to black, although the smaller ones may be white or gray; cystin, pale yellow; and xanthin, bright brown.

Surface Appearance. Oxalate calculi are rough or tuberculated (mulberry stones); urate calculi are slightly rough. Phosphate calculi have a smooth or sandy surface; cystin and xanthin stones are usually smooth.

Consistence. Cystin and phosphate stones are the softest; the former are waxlike, the latter earthy or chalky and somewhat brittle. Urate stones are much harder, and oxalate calculi are the hardest.

Chemical Examination of Calculi

The larger stones should be sawed into two pieces, the cut surfaces smoothed and washed with water. If it is possible to distinguish a distinct nucleus surrounded by concentric layers, split off the successive layers with a knife, and examine the nucleus and each layer separately. If the stone appears homogeneous, crush it and grind it up to a fine powder in a mortar.

Transfer a portion of the powder to a piece of platinum foil and heat.

A. If the powder burns up entirely or leaves only a slight residue it is composed of organic materials (uric acid, urates, cystin, xanthin).

Urate and xanthin stones burn without a flame and give off an odor of prussic acid. Cystin stones burn with a bluish flame and give an odor of sulfuric acid. To differentiate:

1. Moisten a second portion of powder with nitric acid and evaporate it to dryness. If the addition of a drop of ammonia to part of this dried residue gives a purple-red color, and if a drop of sodium hydroxide gives a blue-violet color, the stone contains uric acid or urates (murexide test).

2. If the addition to the original powder of a drop of sodium hydroxide yields ammonia, the stone contains ammonium urate. If it does not do this and burns completely with a glow, it is pure uric acid. Other urates yield a slight residue.

3. If the addition of ammonia to the dried powder gives no color, but if sodium hydroxide gives a bright-red color, the stone is of xanthin.

4. Cystin stones give no color with either. They dissolve easily in ammonia and separate from the solution as hexagonal plates when the ammonia evaporates.

B. If the specimen does not burn appreciably, or if it only blackens and (after glowing) leaves a considerable residue, it is composed largely of phosphates, carbonates and oxalates.

1. Dissolve the residue (or some of the original powder) in hot dilute hydrochloric acid. (Any organic matrix or uric acid present will not dissolve.) Cool and clear by centrifugation or filtration and test the insoluble portion for uric acid by the murexide test.

2. Dilute the filtrate with a little water and add ammonia until it is strongly alkaline. The formation of a precipitate may be due to (*a*) earthy phosphates (calcium and magnesium phosphates, or triple phosphate—ammonium magnesium phosphate) or (*b*) calcium oxalate.

3. Remove the precipitate by centrifugation, and to it add acetic acid. The earthy phosphates dissolve. Calcium oxalate does not dissolve and can be identified by the shape of the crystals.

4. To the acetic acid solution add ammonium molybdate and nitric acid, and heat the mixture to 60° C. The formation of a yellow precipitate indicates the presence of phosphoric acid.

5. If no precipitate forms upon the addition of ammonia in (2), the calculus is either calcium or magnesium carbonate. To a bit of the calculus add a drop of hydrochloric acid. The evolution of gas (CO_2) proves the presence of carbonates.

6. To half the ammoniacal solution add ammonium oxalate solution. The formation of a precipitate (of calcium oxalate crystals) shows the presence of calcium carbonate.

7. To the other half of the ammoniacal solution add sodium phosphate solution. The formation of a precipitate (of triple phosphate crystals) shows the presence of magnesium carbonate.

Chemical Tests of Urine

Tests for Protein (Serum Albumin and Serum Globulin). The urine must be clear. If cloudy, it must be filtered, after shaking it up with Kieselguhr if necessary.

Heat and Acetic Acid. A thin-walled test tube is two-thirds filled with *clear* urine,

and the upper portion (about 1 inch in depth) is brought to gentle boiling over a free flame. A few (3 to 10) drops of 5 per cent acetic acid are added drop by drop, and the boiling continued. A cloud which appears on boiling and disappears on addition of acid is due to carbonates and phosphates. A cloud which persists or increases in density after acidification is usually due to albumin.

A positive reaction may also be given by *mucin* ("nucleo-albumin"), a substance which may be secreted by the mucous membranes of the urinary passages, particularly if slightly inflamed or irritated. It may be differentiated from albumin by the fact that it is precipitated by acetic acid in the cold. It has no clinical significance.

Interference by mucin can be avoided and the test made more delicate by adding to the urine one-fifth its volume of saturated sodium chloride, and 2 to 5 drops of glacial acetic acid before boiling (Purdy). We regard the test thus modified as the most practicable and delicate of the ordinary procedures for routine work.

The approximate strength of the reaction may be expressed as follows: Trace: a faint cloud, clearly seen only when viewed against a dark background, best with illumination from above. $+^1$: a cloud which is distinct, but still transparent. $+^2$: a uniform opaque cloud. $+^3$: a dense cloud which does not immediately flocculate. $+^4$: immediate formation of a flocculent coagulum.

Nitric Acid Test (Heller). In the bottom of a conical glass or wide test tube put a few ml. of concentrated nitric acid, or preferably Robert's reagent (HNO_3, 100 ml.; saturated aqueous solution of $MgSO_4$, 500 ml.). Over this layer carefully a few ml. of clear urine, by inclining the tube and allowing the urine (from a pipet) to flow slowly down the side of the tube. Examine after three minutes. In the presence of albumin an opaque white cloud ("ring") forms at the plane of contact. In the absence of albumin confusing rings may be formed at the surface of contact by urea nitrate crystals (avoided by diluting the urine), and by resins (soluble in alcohol). Rings above the line of contact may be formed by urates, or mucin. Colored rings without turbidity, due to oxidation of pigment or ingested drugs, should not be confusing. This test is less sensitive than the preceding one, but useful when only small amounts of urine are available. Urine containing 0.0033 per cent albumin will give a perceptible ring in exactly two minutes. A quantitative estimate can be made by determining the dilution of urine with 0.6 per cent salt solution which will just give a reaction in two minutes, and multiplying this by 0.0033 per cent.

Quantitative Estimation: with esbach's albuminometer. A rough quantitative estimation may be made with Esbach's albuminometer when considerable albumin is present. The clear urine is added to the mark U; if not strongly acid, 1 or 2 drops of glacial acetic acid is added; and the tube is filled to the mark R with Tsuchiya's reagent (phosphotungstic acid, 1.5 Gm.; HCl, 5 ml.; 95 per cent alcohol, 85 ml.). The tube is mixed by gentle inversion several times, and allowed to stand just 24 hours. The depth of the precipitate is read on the graduated tube, and gives directly the protein content in Gm. per liter.

purdy's method. Put 10 ml. urine in a graduated centrifuge tube; add 2 ml. of 50 per cent acetic acid, then 3 ml. of 10 per cent potassium ferrocyanide. Mix by gentle inversion, and after standing 10 minutes, centrifuge exactly three minutes at 1500 revolutions per minute in a centrifuge having a radius of 6¾ inches with tubes extended. Albumin (in per cent by weight) = 0.21 × volume of sediment in ml.

sulfosalicylic acid method. The association of Life Insurance Medical Directors of America have adopted the following method. In a test tube graduated at 10 ml., pipet 2.5 ml. clear urine, fill to the mark with 3 per cent sulfosalicylic acid, and mix by inversion. After 10 minutes compare the turbidity with that of standard tubes of known protein content. Standards that are permanent for at least eight months can be made by the following method (Kingsbury, 1926) or can be purchased (see also p. 749).

Gelatin Solution. Dissolve 50 Gm. gelatin (Difco) in about 350 ml. distilled water at

50° C., and dilute to 500 ml. with water. This may be stored for a few days (only) in the icebox.

Formazin Suspension. Dissolve 0.25 Gm. hydrazine sulfate in 25 ml. distilled water. Dissolve 2.5 Gm. methenamine in 25 ml. distilled water, and pour this slowly into the hydrazine sulfate solution, stirring constantly. Stopper and let stand 18 to 24 hours.

Standards. To 200 ml. of the 10 per cent gelatin solution, warmed to 40° to 45° C., add 0.6 ml. of 40 per cent formaldehyde and 29 ml. freshly agitated, warm formazin suspension, and mix. In a series of large tubes or flasks prepare mixtures of formazin-gelatin suspension and 10 per cent gelatin solution in the quantities indicated in the following table.

Table 77

QUANTITATIVE ESTIMATION OF PROTEIN IN URINE—KINGSBURY'S STANDARD

Formazin-gelatin Suspension ml.	*10% Gelatin ml.*	*Turbidity Corresponds to:*	
		Protein in mg. per 100 ml.	*Protein in per cent*
50	0	100	0.10
37.5	12.5	75	0.075
25	25	50	0.05
20	30	40	0.04
15	35	30	0.03
10	40	20	0.02
5	45	10	0.01

Select tubes identical in diameter with those used in examining the urine, pour 10 ml. of each standard into one of these tubes, stopper tightly, chill in cold water to solidify the gelatin, and store in a room cool enough to prevent melting of the gelatin.

NOTES. Although it is important to have a rough estimate of the amount of albumin present, the additional information gained by precise estimations rarely repays, for practical purposes, the time spent in making them.

The use of more sensitive reagents such as trichloracetic acid for routine purposes is undesirable, as they often give a positive reaction with normal urine, and may be confusing.

Proteoses, which are protein decomposition products, may appear in the urine in conditions in which necrotic tissue or inflammatory exudates are being absorbed. They are precipitated by sulfosalicylic acid, trichloracetic acid, and in part by cold nitric acid, but not by heat and acetic acid. Care must be taken to distinguish them from albumin if the former reagents are used, as their significance is entirely different.

To *remove albumin* as a preliminary to other tests, put a measured volume of urine in an Erlenmeyer flask, acidify slightly with dilute acetic acid if not acid to litmus, and bring to boiling. In case the albumin does not separate as a flocculent precipitate (but merely causes a diffuse turbidity), if the specific gravity is low, add a few ml. of saturated sodium chloride solution, and again boil. If necessary add more dilute acetic acid, drop by drop, boiling after each addition. Excess of acid must be avoided. Filter until clear, and test filtrate for albumin, preferably with sulfosalicylic acid. Dilute to original volume.

SIGNIFICANCE. A positive reaction for albumin by the usual tests is probably always pathologic, but it may occur as a result of clinically insignificant or transient causes, and by no means necessarily indicates kidney disease. The significance of albuminuria depends entirely upon the condition causing it. This can only be

determined by a clinical study of the patient in conjunction with the results of urine examinations, and rarely by the latter alone.

Albumin may enter the urine through the kidney, or at any point in the urinary passages ("postrenal albuminuria"), usually as a result of local bleeding, irritation, or infection. Albumin may appear in the urine as a result of irritation or disease of the kidney (renal albuminuria) or of circulatory disturbances or other extraneous conditions ("prerenal albuminuria"). In either case casts are usually present also, although there is no close parallelism between the number of casts and the amount of albumin present. Among the causes of prerenal albuminuria are congestion resulting from myocardial failure, or pressure on the renal veins by a pregnant uterus, or a tumor; severe anemia; and probably excessive muscular exercise, and cold baths.

The *orthostatic albuminuria* of adolescents probably belongs in this group. It is characterized by the excretion of albumin, often in large amounts, while the patient is active in the erect posture, and complete disappearance of the albumin while prone in bed. No casts or blood cells are present, and renal function is normal while prone, but in the erect posture renal epithelial cells and casts (hyaline or more often granular) may be excreted, and renal function may be impaired, as determined by the more sensitive tests (Rytand, 1937). Many cases show an exaggerated lumbar lordosis, and pressure on the renal veins has been suggested as an explanation. Others attribute it to circulatory disturbances associated with vasomotor instability and a low pulse pressure. In its slighter degrees it probably accounts for most of the "physiologic" or benign albuminurias of adolescence. The prognosis is usually good, although the diagnosis must be made with caution, since posture may have a marked influence on the albumin output in true nephritis.

Renal albuminuria may be due to irritation from various drugs and poisons, notably mercurial salts, lead, phenol, turpentine, cantharides, mustard. It also occurs frequently in many acute febrile diseases, such as typhoid fever, pneumonia, and scarlet fever and other streptococcal infections, without evidence of a true diffuse nephritis. It is practically constant in all forms of nephritis, including the focal nephritis of chronic sepsis, the arteriosclerotic nephropathies, amyloid kidney, and chronic pyelonephritis, as well as acute and chronic glomerulonephritis, including the "nephroses." In acute nephritis the quantity of albumin varies with the acuteness and severity of the process, is usually large, and with the presence of red blood cell casts, makes the diagnosis certain. Extremely large amounts of albumin (20 to 80 Gm. per liter) are seen chiefly in "nephrotic" types of nephritis, including syphilitic nephritis. In other cases there is no definite relation between the gravity of the disease and the degree of albuminuria. Inconstant traces only may be excreted in advanced stages of contracted kidney. The presence of epithelial casts and coarsely granular casts indicates active disease of the kidney with injury to the tubular epithelium, usually a nephritis (particularly if red cells are also found).

It has not been possible to make a classification of cases of nephritis which harmonizes both with the anatomic changes in the kidney and the clinical manifesta-

Table 78

CLINICAL TYPES OF RENAL DISEASE

	Urine	*Specific Gravity*	*Albumin*	*Sediment*	*Etiology*	*Special Features*
Acute glomerulonephritis (Exacerbations of chronic glomerulonephritis)	Diminished. Color dark. Hematuria frequent	High, 1.025–1.030	Abundant	Abundant. Hyaline, granular, cellular, red cell casts. Red and white cells. Renal epithelium	Follows acute infections, nearly always streptococcal	Abrupt onset. Edema frequently marked, especially of face; may be trivial. Hypertension, nitrogen retention common. May recover, become chronic, or terminate in uremia
Nephrotic stage of active glomerulonephritis (Includes some cases of "nephrosis")	Usually diminished. Color moderately dark	High, 1.020–1.030	Very large amount; 0.5 to 8.0%	Abundant as above, often fatty and waxy casts. Red cells often scanty. Doubly refractile granules often found with polarizing microscope	Follows acute infections	Edema marked. Low-plasma proteins. Degenerative changes marked. Blood pressure usually normal. Uremia not common. Merges with (1), may terminate in (3)
Chronic glomerulonephritis	Usually increased, pale, clear. Nocturia marked	Usually low, often fixed under 1.010 –1.012	Usually small amount or trace only	Scanty. A few hyaline and granular casts and a few red cells	Repeated acute or chronic infections. Subject to acute exacerbations	Usually no edema. Hypertension and nitrogen retention usual. Albuminuric retinitis. Terminal uremia
Focal nephritis	Normal in amount. Periods of hematuria	Normal	Slight to marked	Red cells. Variable number of casts	Sepsis, especially subacute bacterial endocarditis	No edema. No hypertension or uremia. Retinal hemorrhages
Arteriosclerotic nephropathy. Vascular nephritis	Normal or increased. Often nocturia	Normal or usually diminished	Trace or small amount	Scanty. A few hyaline casts. Red cells few or absent, rarely found	Not known. In part heredity, obesity, nervous tension, wear-and-tear of life	Hypertension and cardiac hypertrophy marked. Death usually from cardiovascular disease. Nitrogen retention and uremia may occur late. Arteriosclerotic retinal changes
Chronic passive congestion	Diminished. Color dark	High	Slight or moderate	Variable. Hyaline and granular casts. May show a few red cells. Sedimentum lateritium	Myocardial insufficiency from any cause	No uremia or nitrogen retention unless complicated. Salt excretion impaired. "Cardiac" edema, normal plasma proteins
Pyelitis and cystitis	Normal amount. Turbid in varying degree	Normal	Slight to marked	Pus in varying amount. Some red cells. Bacteria. Often a few	Descending or ascending infections of urinary tract	No edema. Common with urethral or ureteral obstruction. May lead to hydronephrosis, pyelonephri-

tions of the disease, nor to predict with any constancy from the latter, just what the autopsy findings will be. Table 78 shows the usual major features in typical cases of the main clinical types of nephritis. Many transitions and combinations occur.

The term "nephrosis" has been used for the sake of brevity in these discussions as a synonym for "acute degenerative nonhemorrhagic nephritis with edema," the so-called lipoid nephrosis. The term is a misnomer as applied to these cases, since recent work has shown that there are invariably some inflammatory changes in the kidneys, and the condition cannot be differentiated sharply from ordinary acute hemorrhagic nephritis with edema.

Bence-Jones Protein. This may be demonstrated by acidifying a clear specimen of urine with dilute acetic acid, and heating gradually in a water bath. A turbidity or precipitate which is maximal at 60° C., and which dissolves on boiling and reappears on cooling, indicates its presence. If albumin is also present it may be removed by filtering the hot urine (not always successful). The Bence-Jones protein, if present, will reprecipitate on cooling. The substance appears in many (not all) cases of multiple myeloma; and rarely in leukemia and in carcinoma or other malignant growths invading the bone marrow.

Congo Red Test for Amyloidosis. (1) Inject intravenously 0.25 ml. per kilogram of body weight (not over 18 ml.) of a 1.5 per cent solution of Grübler's Congo red. (See Blood Volume, p. 398.) (2) Four minutes later withdraw 10 ml. of blood from the opposite arm with a clean syringe and oxalate in a graduated centrifuge tube, taking the usual precautions to prevent hemolysis. (3) One hour after the injection similarly obtain and oxalate a second 10-ml. specimen of blood. Also secure a specimen of urine. (4) Centrifuge both the four-minute and one-hour specimens until the plasma is clear. (5) Transfer the plasma to colorimeter cups. (6) Set the cup containing the four-minute plasma at 10 mm. (regarded as the 100 per cent standard), and match the other against it.

$$\text{The per cent of dye retained} = \frac{\text{Reading of 4-minute plasma}}{\text{Reading of 1-hour plasma}} \times 100.$$

The per cent of dye eliminated is obtained by subtracting this figure from 100 per cent.

In normal cases less than 30 to 40 per cent of the dye is eliminated (usually less than 20 per cent). In "nephrosis" from 40 to 60 per cent may be eliminated, but a considerable amount of the dye will be found in the urine, which will be deep red in color. The elimination of more than 40 per cent (usually more than 60 per cent) of the dye from the blood without the appearance of much dye in the urine usually indicates amyloidosis. The result of the test may be misleading, however, in individual cases.

Sugar. Reducing substances, including some fermentable carbohydrates, are present in traces (0.1 per cent or less) in normal urine. The amount of glucose appears to be very minute. In pathologic conditions larger amounts of glucose may be present; rarely also lactose, levulose, maltose, and certain pentoses. All these sugars reduce alkaline copper solutions. Preservatives interfere with some of the tests. Albumin, if present, should be removed.

Benedict's Qualitative Test. This is the best for routine use, as it is more sensitive, and gives fewer positive reactions with other reducing substances than do other methods. It is not reduced by formaldehyde or chloroform.

To make the solution dissolve 173 Gm. sodium citrate and 100 Gm. anhydrous sodium carbonate (or 200 Gm. crystalline) in 700 ml. water (boiling-water bath), and filter. Add slowly, with constant stirring, 17.3 Gm. copper sulfate dissolved in 100 ml. water, cool, and dilute to 1 liter. It keeps indefinitely.

Procedure. (1) Put exactly 5 ml. of the solution in a test tube, and heat to boiling

(to check solution). (2) Add 0.5 ml. urine (8 to 10 drops only) from a pipet. (3) Mix well, and put in boiling-water bath for five minutes or boil vigorously over a free flame for one to two minutes. (4) Remove and let cool *slowly*. A positive reaction is indicated by a general turbidity, due to the formation of a bulky precipitate which is greenish, yellow, or brick-red, according to the amount of sugar present; and, later, by the deposition of a yellow or red precipitate. A whitish turbidity or sediment is due to urates and is of no significance. A weak reaction may be evident only after standing. The test will detect glucose in concentration of 0.02 per cent. A positive reaction is not specific for glucose but merely proves the presence of some reducing substance. Weak and usually somewhat atypical reactions may be obtained after the administration of many different drugs. When a reducing substance is found *for the first time* in the urine, it must be further identified by the fermentation test, and if the reaction is positive, preferably also by the phenylhydrazine test.

Fermentation Test. (1) Boil about 25 ml. of urine (free from preservative) to kill colon bacilli which may ferment lactose if that be present, and cool. (2) Rub up a piece of fresh yeast the size of a pea in the urine, and acidify faintly with tartaric acid, if necessary. (3) Put in fermentation tube, taking care that all air bubbles are expelled, and incubate a few hours. (4) If not certain that the yeast is active and free from fermentable sugar, similarly set up controls of yeast in normal urine; and yeast in normal urine to which glucose has been added in about the same percentage as that present in the specimen which is to be examined.

A positive reaction is indicated by the appearance of gas in the closed arm of the tube about equal in volume to that in the tube to which glucose was added; or if less than about 0.1 per cent of glucose was present, merely by the failure of the fermented urine to reduce Benedict's solution.

Although this test may (rarely) give false positive reactions, in conjunction with a positive Benedict's test it practically proves the presence of glucose. Levulose, and galactose and maltose (slowly) react similarly. Their differentiation from glucose is difficult and rarely of any importance, since practically they only occur with glucose in diabetes. Lactose and pentoses are not fermented. Glucose is the only reducing substance which produces a hyperglycemia.

Phenylhydrazine Test (Kowarski). This is not a practicable procedure for the detection of glucose, but is of value in identifying it conclusively. (1) In a test tube put 5 drops basic phenylhydrazine, (2) 10 drops glacial acetic acid, and (3) 1 ml. saturated sodium chloride solution. (4) Add 4 to 5 ml. of filtered urine and an equal volume of distilled water, insert a small piece of glass tubing to lessen bumping, and boil over a free flame with constant shaking until the volume is reduced to about 3 ml. (5) Put in hot-water bath and cool slowly without agitation. (6) Examine under the microscope for glucosazone crystals, which appear as fine straight needles arranged in fans, sheaves, or spherical clusters. Their appearance is practically pathognomonic. After purification by recrystallization from hot 60 per cent alcohol, they melt at about 200° C. Only a positive reaction is significant.

Levulose yields the same osazone. Similar osazones may be obtained from lactose, galactose, and maltose, but only under special favorable conditions, very rarely in urine. They can be differentiated from glucosazone crystals by their shape, maltose yielding broader, blunter crystals, and lactose, finer, curled crystals. Pentoses yield osazones melting at about 165° C., and glycuronic acid an osazone melting at 115° C.

Quantitative Estimation of Glucose. A mixed 24-hour specimen should be obtained and its volume noted.

Benedict's quantitative solution is recommended. To prepare the solution (1) dissolve in about 650 ml. of water 100 Gm. anhydrous (or 200 Gm. crystallized) sodium carbonate, 200 Gm. sodium (or potassium) citrate, and 125 Gm. potassium thiocyanate. Filter if necessary. (2) In 100 ml. of water dissolve exactly 18.0 Gm. copper sulfate (crystals). (3)

Pour slowly, with constant stirring, into the above solution. (4) Add 5 ml. of 5 per cent potassium ferrocyanide solution. (5) Cool, dilute to 1 liter, and filter if necessary. It keeps indefinitely. Twenty-five ml. are reduced by 0.050 Gm. glucose.

PROCEDURE. (1) Dilute the urine 1 : 10 unless the amount of sugar is small. (2) To 25 ml. of the reagent in a 500-ml. Erlenmeyer flask, add 10 Gm. anhydrous sodium carbonate and a little powdered pumice stone, and bring to a boil. (3) From a buret add the diluted urine rapidly until a chalky white precipitate begins to form, then more slowly, a few drops at a time, until the blue color has disappeared. The flask must be kept boiling, and be constantly agitated and water lost by evaporation must be replaced.

Calculation:

$$\frac{0.050 \times 100}{\text{No. ml. of urine}} = \text{Per cent glucose.}$$

If only small amounts of urine are available, 5 ml. of reagent may be used, in a 100-ml. flask.

Calculation:

$$\frac{0.010 \times 100}{\text{No. ml. of urine}} = \text{Per cent glucose.}$$

Any other reducing substances present are included with glucose in these measurements.

SIGNIFICANCE. Recent work indicates that the fermentable reducing substances in normal urine include only insignificant traces of glucose (less than 0.01 per cent), and consist largely of unutilizable carbohydrates from the food. The normal individual does not excrete appreciable amounts of glucose until the blood sugar rises above a definite *"rising threshold" level.* This varies with different individuals from 114 to 216 mg. % in arterial blood (Höst), but is constant in the same individual. If it rises much above this level and if the rise is maintained for an appreciable time, considerable amounts of glucose will be excreted. Once initiated, the excretion of glucose continues until the blood sugar has fallen to a level considerably below that at which excretion began—the *"falling threshold" level.* This also varies greatly and may be at or near the normal fasting blood-sugar level.

Glycosuria may occur in a normal individual as a result of any of the factors which may elevate the blood sugar, enumerated on p. 775, provided the rising threshold level is exceeded. It is frequently seen as a result of powerful emotional disturbances. It occurs in some normal persons after a heavy carbohydrate meal, although in most normal individuals it is difficult or impossible to administer enough carbohydrate (as starch or glucose) by mouth to produce a glycosuria.

Glycosuria may occur in a variety of pathologic conditions other than diabetes. The most important ones have already been enumerated as causes of hyperglycemia (see p. 775). The possible presence of these conditions must always be considered, but a persistent glycosuria associated with a high fasting blood sugar in an individual on an ordinary diet usually means diabetes mellitus. Absence of a glycosuria does not exclude diabetes. Apart from the effect of dietary restriction, elderly diabetics with considerable arteriosclerosis may show little or no

sugar in the urine, although their fasting blood sugar is 200 mg. % or even higher (high threshold).

Lactose is often excreted in the urine of lactating women and occasionally in sucklings. *Pentoses* may be excreted after eating large quantities of fruit. They are also met with in a rare constitutional anomaly, *"pentosuria,"* a condition which does not affect the health but may be mistaken for diabetes. They are nonfermentable. *Galactose* is rarely found except after its administration as a test of liver function. *Glycuronic acid compounds* are excreted in minute amounts in normal urine, but they may be increased greatly after taking certain drugs (chloral, camphor, morphine, salicylates, etc.). They may be mistaken for glucose as they will reduce Benedict's solution, but are not fermentable. Another nonfermentable reducing substance is *homogentisic acid,* excreted in the rare constitutional anomaly, *alkaptonuria.* Urine containing it blackens on standing, from oxidation.

Ketone Bodies. Acetone, diacetic acid, and β-hydroxybutyric acid are present in large quantities in the urine (ketonuria) in cases of diabetic acidosis. Smaller amounts of acetone and diacetic acid may be present in normal individuals or in other conditions if the individual is starved or if on a diet which is very high in fat and low in carbohydrate. Ketonuria indicates incomplete oxidation of fats. The former view, however, that this is due directly to the inability of the body simultaneously to oxidize glucose has been largely abandoned. The demonstration of acetone and diacetic acid in the urine in diabetes is of great practical value in indicating the presence of acidosis. If the reaction for diacetic acid is strong, β-hydroxybutyric acid may safely be assumed to be present also. However, quantitative estimations of these substances are less valuable in measuring the degree of acidosis than are other simpler procedures (e.g., the CO_2 combining capacity of the plasma) and are rarely worth the time required to do them.

There are other types of acidosis in which ketonuria does not occur.

Rothera's Test for Acetone. To 3 ml. urine add an excess of ammonium sulfate and shake until saturated. Add 3 drops of fresh 5 per cent solution of sodium nitroprusside, and layer over this 1 to 2 ml. concentrated ammonia. A positive reaction is indicated by a reddish-purple band appearing within 15 minutes. The test is delicate, and like all clinical tests for acetone reacts also with diacetic acid. A weak positive reaction has little significance.

If a positive reaction is obtained, test for diacetic acid by Gerhardt's method. (Urine which gives a negative reaction to Rothera's test will never give a positive reaction to Gerhardt's test.) The urine must be fresh, as diacetic acid is converted into acetone on standing.

Gerhardt's Test for Diacetic Acid. To 5 ml. urine add 5 per cent ferric chloride drop by drop until the precipitate which forms redissolves. In the presence of 0.05 per cent diacetic acid a deep-red color develops. As a control, repeat the test with a specimen which has been boiled to drive off the diacetic acid. (If a red color then develops it is due to other substances, which occur commonly after taking salicylates and related drugs, sodium bicarbonate, etc.) This test is not very delicate. A positive reaction indicates a significant degree of acidosis.

If there is doubt as to the outcome of these reactions, acidify 250 ml. urine slightly with phosphoric acid and distill off 20 ml. of fluid.

Apply Rothera's test to the distillate. All diacetic acid is converted into acetone, and disturbing substances are eliminated.

Ketogenic Diet. An artificially produced ketosis by means of a diet high in fat and very low in carbohydrate has been utilized for the treatment of epilepsy and migraine, and also for urinary-tract infections. In epilepsy a moderate ketosis is maintained over a long period, whereas in infections of the urinary tract an abrupt and intense ketosis should be developed over a period of about 10 days. The introduction of the sulfonamides and of penicillin, however, has greatly limited the need for this procedure.

Bile Pigments. Bilirubin and bile salts are present in the urine in any condition causing obstructive jaundice. The obstruction may be only partial, and may be due to disturbances within the liver, as well as in the ducts. Bilirubin may be demonstrable in the urine when there is no visible tingeing of the skin or sclerae.

Foam Test. On shaking the urine, the foam shows a yellow color. (Not sensitive, or specific.)

Gmelin's Test (Rosenbach's Modification). (1) Shake up the urine and filter a considerable quantity through a small filter. (2) Dry paper and sediment, in air. (3) Add a drop of yellow nitric acid to paper. A positive reaction is indicated by a play of colors: first, green, then various shades of red and blue. It is better to apply the test to the (dried) precipitate obtained when bile is removed as a preliminary to Schlesinger's urobilin test, q.v. (Not sensitive, fairly reliable if positive.)

Alcoholic Iodine Reaction. Over the urine, layer a 1 per cent alcoholic iodine solution. If positive, a green band appears. (Simple, fairly sensitive and reliable.)

Huppert-Nakayama Reaction. (1) In a centrifuge tube put 5 ml. urine and 5 ml. 5 per cent barium chloride solution. (2) Centrifuge two minutes. (3) Decant and discard the fluid. (4) Add to the precipitate 2 ml. ferric chloride reagent, mix, and heat just to boiling (about 10 seconds in a boiling-water bath). If positive the fluid turns green or bluish green. (5) Add 1 drop of yellow nitric acid. If positive the color changes to violet and red.

reagent. To 990 ml. alcohol add 4 Gm. ferric chloride and 10 ml. concentrated hydrochloric acid, and heat to boiling.

The test is sensitive (1 : 1,000,000), and reliable if a negative reaction is obtained with normal urine run in parallel as a control.

Bile Salts: Hays Test. Put the cool urine in a beaker, and sprinkle finely powdered sulfur on the surface. If bile acids are present in concentration of 0.01 per cent, they lower the surface tension, so that the sulfur sinks at once. If it floats even after gentle shaking, the reaction is negative. (Sensitive but not specific.)

Urobilinogen and Urobilin. Urobilinogen and urobilin are present in small amounts in normal urine. They are absent in patients with complete obstruction of the common bile duct. They are increased in conditions associated with increased blood destruction, or with diffuse liver injury. In freshly voided urine pigments are largely or entirely in the form of urobilinogen, which is rapidly converted into urobilin by light and acid.

Urobilinogen Test. To a small amount of *fresh* urine add one-tenth its volume of Ehrlich's reagent (2 per cent solution of paradimethylamidobenzaldehyde in 20 per cent hydrochloric acid). Let stand 10 minutes in the dark. A normal urine shows a distinct but rather faint reddish tinge (much intensified by heating). When urobilinogen is increased, a deep, bright-red color rapidly develops. If more than a faint color is present set up a series of graded dilutions of urine (2 ml. each in small test tubes) from 1 : 10 to 1 : 200; add 0.2 ml. of reagent (do not heat); after five minutes inspect by looking down

through the fluid against a white background. Note the highest dilution giving a distinct pink color. The reaction of normal urine is negative in dilutions higher than 1 : 10.

Schlesinger's Test for Urobilin. (1) If bile is present, first remove it by adding one-fifth volume of 10 per cent calcium chloride, and one-fifth volume of saturated calcium carbonate solution, and filtering. (2) To 10 ml. add a few drops of iodine solution (e.g., Lugol's), to convert all the urobilinogen and (3) an equal volume of saturated alcoholic zinc chloride solution (or 10 per cent alcoholic zinc acetate solution), mix, and filter. Look for a greenish fluorescence, by holding the tube in bright sunlight against a black background, focusing the rays on the tube with a lens. The fluorescence gradually increases on standing. In normal urine only a faint tinge can be detected. In pathologic urine there may be a deep-green color. A rough quantitative test may be done by determining the highest dilution at which the greenish fluorescence can just be seen (for most workers, the end point is harder to determine than with urobilinogen). The method of Elman and McMaster may be used (p. 855). The solutions give a characteristic absorption band in the blue-green (see p. 813). If absence of urobilin is to be demonstrated, a considerable volume of urine must be extracted with chloroform, and the alcoholic zinc chloride solution added to the extract.

Indican. (1) To 10 ml. urine add 2 ml. liquor lead acetate (U.S.P.) and filter. (2) To 6 ml. of filtrate add an equal volume of Obermeyer's reagent (0.1 Gm. $FeCl_3$ in 50 ml. of HCl). (3) After five minutes add 2 ml. chloroform and mix. If indican is present, the chloroform absorbs a blue color from the urine. Its presence is attributed to intestinal stasis. Its demonstration has little practical significance.

Hemoglobin: Orthotolidine Test. (1) Centrifuge 15 ml. urine for five minutes at 1500 revolutions per minute. (2) Decant the supernatant fluid. (3) To the sediment add 2 drops of orthotolidine solution and 2 or 3 drops of acid peroxide solution.

A positive reaction is indicated by the appearance of a greenish-blue to a deep-blue color lasting one to two minutes. A positive reaction is given by a sediment containing 100 red cells per cu. mm. This test, however, is not specific for hemoglobin.

Solutions: (1) A 1 per cent solution of orthotolidine in methyl alcohol. This keeps a year.

(2) Glacial acetic acid, 1 part; hydrogen peroxide, 2 parts. This keeps several months.

Benzidine Test. The benzidine test as outlined under Examination of Feces may be used.

Spectroscopic Examination. If the filtered urine is reddish tinged, it may be examined spectroscopically, for hemoglobin (see p. 813). If positive this test is specific, but it is not delicate.

Diazo Reaction. To a little urine add an equal volume of Ehrlich's diazo reagent (see p. 872). Shake vigorously and add 2 ml. ammonia. A positive reaction is indicated by a bright-red color (not yellow or orange) in the urine and particularly in the foam. The presence in the urine of certain aromatic substances, which are responsible for the positive reaction, in some severe acute infections, chiefly typhoid fever, measles, and acute tuberculosis, is a distinctly unfavorable sign.

Demonstration of Mercury in Urine (Perlstein and Abelin). To 500 ml. urine add 3 ml. of egg albumin which has been brought into solution by trituration with a little of the urine. Boil and collect the precipitate on a filter. Dry the precipitate between sheets of filter paper and rub up in 10 ml. of concentrated hydrochloric acid. Into the acid put a small piece of clean copper foil or wire (2 inches of wire coiled up) and let stand over night. Remove the wire, using forceps, wash in water, alcohol, and ether, and dry. Put in a small, clean, *dry* test tube or sealed glass tube and heat gently until the copper has a grayish color. Remove the copper, introduce a small crystal of iodine, and warm gently. If mercury is present a red precipitate of mercuric iodide forms on the wall of the tube. The reaction is more easily seen if a small plug of dentists' gold leaf is inserted in the tube near the copper before heating. If mercury is present, a small silvery spot of amalgam

forms on the gold near the copper, and this turns red in the presence of iodine vapor. The test will detect 0.1 mg. of Hg in 500 ml. of urine.

Arsenic (Gutzeit's Method). To 5 ml. urine in a large test tube add 5 ml. dilute hydrochloric or sulfuric acid, a few drops of iodine solution, and a few small pieces of zinc, all arsenic-free. Immediately put over the mouth of the tube a piece of filter paper moistened with saturated silver nitrate solution. If arsenic is present, within 10 to 15 minutes the paper will show a lemon-yellow color (silver arsenide). This will turn black if moistened with a drop of water, or more gradually as it dries in the air. Interfering substances are rare.

Lead (Klopstock and Kowarski). Concentrate a 24-hour specimen of urine on a water bath to one-fifth its volume, add an equal volume of concentrated hydrochloric acid, heat to boiling, and add potassium chlorate, a little at a time, until the fluid becomes colorless. Boil off the excess chlorine and add sodium carbonate until nearly neutralized. Filter and pass hydrogen sulfide through the filtrate. If a blackish precipitate forms, collect it on a small filter paper and wash. Transfer the paper and precipitate to a small beaker, dissolve precipitate in a little hot dilute nitric acid, dilute with water, and filter. Evaporate to dryness and redissolve in a little water. Test portions for lead as follows: (1) Dilute sulfuric acid produces a white precipitate; (2) potassium chromate, a yellowish; (3) potassium iodide, a yellowish; and (4) hydrogen sulfide, a black precipitate. Lead may be determined quantitatively by Fairhall's method (*J. Biol. Chem.*, 60).

Special Quantitative Tests

Total Solids. Total solids may be estimated roughly in Gm. per liter by multiplying the last two digits of the specific gravity figure by 2.6 (1.6 in small children), and the product by the 24-hour urine volume in ml. divided by 1000.

Determination of Nitrogenous Constituents. In all cases a mixed 24-hour collection of urine should be used. If albumin is present in the urine it should be removed (see p. 828). The distilled water must be ammonia-free. The reagents are the same as those described in Chapter 36 for use in blood examinations. In all cases in which there is a marked discrepancy between the color of the solutions to be compared the test must be repeated, using larger or smaller amounts of urine, and the final calculations correspondingly adjusted.

Total Nitrogen. (1) Remove albumin from urine if present (see p. 828). (2) Dilute urine: 1 : 50, if the specific gravity is under 1.015; 1 : 100, if 1.016 to 1.030; and 1 : 200 if over 1.030. (3) Pipet exactly 1 ml. diluted urine into a dry Pyrex ignition tube graduated at 20 ml.

Proceed with the digestion exactly as outlined for determination of nonprotein nitrogen in the blood, steps 2 to 16 (inc.) (see p. 767).

Calculation, if the more dilute standard is used, containing 0.06 mg. nitrogen, with the photoelectric colorimeter:

$$\frac{\text{Reading of unknown}}{\text{Reading of standard}} \times 0.06 \times \text{dilution} \times \frac{\text{Volume of 24-hour urine in ml.}}{1000} = \text{Gm. N in 24 hours.}$$

If the stronger standard was used in the comparison, substitute 0.12 for 0.06 in the equation. With a visual colorimeter, the calculation is the same except that the first fraction is inverted.

The normal is 10 to 16 Gm.

Urea and Ammonia Nitrogen. (1) Pipet 5 ml. urine into a 50-ml. volumetric flask; dilute to the mark and mix. (2) Pipet 2 ml. diluted urine into a 125-ml. Erlenmeyer flask, add (3) 15 ml. distilled water, (4) 0.3 ml. urease solution, and (5) 0.5 ml. pyrophosphate buffer. (5) Mix, cover with a beaker, and incubate at 37° C. for 30 minutes. Add (6) 1 ml. of ⅔ N sulfuric acid and (7) 1 ml. of 10 per cent sodium tungstate. (8) Shake

and filter. (9) Put 2 ml. of filtrate into a nonprotein nitrogen tube (5 ml. if the urine was very dilute). (10) Into a similar tube put 2 ml. working standard nitrogen solution, and into a third tube, 4 ml. of this standard solution. To each of these three tubes add: (11) about 10 ml. distilled water, (12) 0.5 ml. digestion mixture, (13) 1 ml. persulfate solution, and (14) 1 ml. gluconate solution. (15) Bring volume to the 20-ml. mark. (16) To each add 5 ml. Nessler's solution. (17) After 10 minutes read in colorimeter.

Calculation: The final dilution of the urine is 1 : 100. If 2 ml. of filtrate was used in step 9, and the weaker working standard was used in the comparison, with the photoelectric colorimeter,

$$\text{Gm. urea and ammonia N per day} = \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times 0.06 \times \frac{100}{2} \times \frac{\text{Volume in ml. of 24-hour urine}}{1000}$$

If a visual colorimeter is used, invert the first fraction, to read: $\frac{\text{Reading of standard}}{\text{Reading of unknown}}$, and complete the calculation according to the equation above.

The normal is usually 10 to 15 Gm. To express in terms of *urea,* multiply the figure for urea nitrogen by 2.14.

For the purpose of determining urea clearance, it has been found that more consistent results are obtained if both urea nitrogen and preformed ammonia nitrogen are determined together, as in the above procedure.

Urea Nitrogen. To determine urea nitrogen alone: (1) Pipet 5 ml. urine into a 50-ml. volumetric flask; dilute to the mark and mix. (2) Pour about 25 ml. of this diluted urine into an Erlenmeyer flask, and add 4 Gm. of dry dust-free permitit, and shake gently for five minutes. (3) Set aside for a few minutes until clear, or centrifuge a little of this if necessary. (4) Pipet 2 ml. of clear diluted urine so treated into a 125-ml. Erlenmeyer flask, and proceed as above, from steps (3) to (17) (inc.). The calculation is the same.

Reagents are identical with those used for determination of nonprotein nitrogen and urea nitrogen in blood (see pp. 768 and 769).

Ammonia. (Folin-Bell). (1) In a 200-ml. volumetric flask put 2 Gm. dust-free permutit, washing down with 5 ml. water, and (2) 2 ml. undiluted urine, washing down with a little water. (3) Shake gently, but continuously, for five minutes, and rinse to bottom of flask with 25 ml. water. (4) Let stand till clear, decant, and discard fluid. (5) Wash permutit with abundant water, and decant three times, discarding washings. (6) Add a little water. (7) In a similar flask put 5 ml. of stock standard nitrogen solution which has been diluted 1 : 10 (containing 0.5 mg. of N). (8) To both flasks add 5 ml. of 10 per cent sodium hydroxide, and quickly dilute to about 150 ml. (9) Add 10 ml. Nessler's solution to both flasks. (10) Dilute to mark and mix. (11) Let stand 10 minutes, and compare in colorimeter. Calculation, since the standard contained 0.5 mg. N:

Standard/Unknown × 0.5 mg. = mg. ammonia N in 2 ml. urine used; and mg. ammonia N per 1 ml. urine = ½ of this figure.

$$\text{Standard/Unknown} \times 0.5 \text{ mg.} \times \tfrac{1}{2} \times \frac{\text{Vol. in ml. of 24-hour specimen}}{1000} = \text{Gm. ammonia N in 24-hour specimen.}$$

The normal figure is 0.5 to 1.2 Gm. per 24 hours.

Uric Acid. (1) Dilute 1 ml. urine to 100 ml. in a volumetric flask. (2) Put 5 ml. diluted urine in a tube graduated at 25 ml. (3) In a second tube put 3 ml. diluted urine and 2 ml. water. (4) In a third tube put 5 ml. standard uric acid solution. To each tube (5) add 10 ml. urea cyanide solution, and (6) 4 ml. uric acid reagent, keeping the tubes vertical. (7) After 15 to 25 minutes dilute to volume, mix, and read in the colorimeter.

Calculation, if tube 1 (the darker) was used in the comparison, with the photoelectric colorimeter:

$$\text{Gm. uric acid per day} = \frac{\text{Reading of unknown} \times .02 \times 100}{\text{Reading of standard} \times 5} \times \frac{\text{Vol. 24-hour urine in ml.}}{1000}$$

$$\text{With visual colorimeter} = \frac{\text{Reading of standard} \times .02 \times 100}{\text{Reading of unknown} \times 5} \times \frac{\text{Vol. 24-hour urine}}{1000}$$

Reagents are identical with those used in blood analyses (p. 772).

SIGNIFICANCE. Ordinarily estimations of uric acid in the urine are of no practical value in diagnosis. The quantity varies greatly (0.5 to 2.0 Gm. per day) with the purin content of the diet. An increase occurs in any condition in which tissue rich in nuclei is disintegrating (leukemia, acute liver degeneration, pneumonia in resolution). In acute gout the excretion tends to be diminished before an attack and to increase after it.

If a tophus can be found the sodium biurate crystals should be examined microscopically and identified by the murexide test.

MUREXIDE TEST. Put a few crystals in an evaporating dish and add 3 drops of concentrated nitric acid. Evaporate to dryness by heating gently. Cool. To the reddish or yellowish residue add a drop of dilute ammonia. A positive reaction is indicated by a purplish-red color which fades on warming. The crystals cannot be identified by their morphology alone.

CREATININE. Creatinine excretion is practically constant in amount for 24-hour periods in the same individual under the same conditions. It, therefore, affords a simple method for determining whether the entire 24-hour specimen has been secured. Folin's method: (1) In a 100-ml. volumetric flask put 1 ml. of *stock* standard solution (containing 1 mg. creatinine). (2) In a similar flask put 1 ml. urine. (3) To both add 20 ml. of a saturated solution of picric acid and 1.5 ml. of 10 per cent sodium hydroxide. (4) After 10 minutes dilute to the mark with water, mix, and read in the colorimeter.

Calculation (visual colorimeter):

$$\frac{\text{Standard}}{\text{Unknown}} \times 1 = \text{mg. creatinine per ml. urine, and } \frac{\text{Standard}}{\text{Unknown}} \times \frac{\text{Vol. in ml. of 24-hour urine}}{1000}$$

= Gm. of creatinine in the 24-hour specimen.

Normal is 1 to 1.5 Gm.

NITROGEN PARTITION. This is determined by calculating the percentage of the total nitrogen in the urine made up by the nitrogen of the urea, ammonia, uric acid, creatinine, and the undetermined residue. The sum of the urea and ammonia nitrogen depends largely upon the amount of protein in the food. In cases on an average diet, this figure is about 10 (to 15) Gm., or 87 (to 90) per cent of the total nitrogen. On a diet minimal in protein it may fall to 0.5 Gm., or 32 per cent of the total nitrogen. (Smith, 1926.) The absolute quantity of the other constituents is little affected, and their percentage figures rise. It is obvious that such determinations are worthless unless the protein content of the diet is precisely controlled, and even then no changes of clinical significance have been demonstrated except in acidosis. Here there may be a marked rise in ammonia at the expense of the urea. (Normal urea N/ammonia N ratio is about 20/1, falling on a very low protein diet to a minimum of 2/3.)

Chlorides (Volhard-Harvey Method): SOLUTIONS: (A) STANDARD SILVER NITRATE SOLUTION. Dissolve 29.06 Gm. silver nitrate in 100 ml. water in a 1-liter volumetric flask. Add 250 ml. concentrated nitric acid and 250 ml. saturated aqueous solution of iron ammonium sulfate; dilute to the mark and mix. One ml. is equivalent to 0.01 Gm. sodium chloride.

(B) STANDARD AMMONIUM SULFOCYANATE SOLUTION. Dissolve about 6.5 Gm. of ammonium sulfocyanate in just 800 ml. water. Titrate this against solution (A), and add the amount of water calculated to be required to dilute it so that 2 ml. is just equivalent to 1 ml. of the silver nitrate standard solution.

PROCEDURE. (1) In a 250-ml. flask put 5 ml. urine, 100 ml. water and 10 ml. silver nitrate solution (A). (2) If the fluid is pinkish, add drop by drop a strong solution of potassium permanganate until the color is dispelled. Let stand 10 minutes or longer. (3) Add 2 ml. nitrobenzene (or chloroform). (4) Add from a buret standard ammonium sulfocyanate solution (B) until the appearance of the first salmon-red tint that persists for several seconds. (If the first drop does so, add an additional 10 ml. of the silver nitrate solution, and proceed.) The red color at the end point lasts a *few seconds only*.

Calculation:

$$\frac{A - \frac{1}{2}B}{5} \times 10 = \text{mg. NaCl per ml. or } (2A - B) \times \text{Output}/1000 = \text{Gm. NaCl in 24-hour specimen.}$$

The usual normal is 10 to 15 Gm., but varies very greatly with the chlorides in the food. Excretion is diminished or suppressed in most conditions in which blood chloride is reduced (pneumonia, pyloric obstruction, etc.).

Sodium Chloride Tolerance Test. Attempts to utilize the rate of excretion of sodium chloride after a test dose of 10 Gm. as a measure of renal function have not yielded results of dependable value, largely because so many extrarenal factors are involved. A normal individual who has been on an average diet will excrete the entire amount within 24 hours, but if the previous diet has been low in salt, all or most of the 10 Gm. may be retained.

Titrable Acidity (Folin). (1) In a 200-ml. flask put 25 ml. urine (carefully preserved). (2) Add 15 Gm. neutral potassium oxalate and 2 drops of 1 per cent phenolphthalein; shake well for two minutes. (3) Titrate to a pink color with N/10 sodium hydroxide.

Calculation:

$$\text{Total acidity of 24-hour urine} = \text{Vol. of 24-hour urine} \times \frac{\text{Titration figure}}{25}$$

In normal individuals it varies greatly with the diet, is usually 200 to 500 (Hawk and Bergeim). It is increased in acidosis (see p. 806).

True Acidity. True acidity is dependent on the hydrogen-ion concentration, and may be determined by the method of Barnett-Chapman (p. 926). The urine should be diluted 1 : 5 with carbon dioxide-free distilled water, and preserved under oil until tested. The usual normal range is from pH 5.5 to 8.0. A rough estimate can be made with nitrazine test paper.

Diastase (Winslow's Method). (1) In a series of 10 small test tubes place 1 ml. of 1 per cent sodium chloride. (2) Add 1 ml. urine to the second tube, mix and transfer 1 ml. of the mixture to the third tube. Continue down the series of tubes, discarding the extra ml. in the last tube. (3) To each tube add 2 ml. of 0.1 per cent soluble-starch solution, and (4) put in water bath at 37° C. for 30 minutes. (5) Add 2 to 3 drops of N/20 iodine solution (or 1 drop of Gram's iodine solution). Determine the tube containing the smallest amount of urine which shows no blue color. The number of units of diastase = 2 ÷ volume of urine (in ml.) in this tube. The normal figure is 8 to 32. Excretion is diminished in nephritis, and much increased (to 100 to 200) in most cases of acute pancreatitis.

Tests of Renal Function

The secretion of urine is a complicated process in which both the glomeruli and the convoluted tubules play essential and independent roles. The production of the glomerular filtrate appears to be a purely mechanical process of ultrafiltration, the glomerular membrane merely holding back the plasma proteins and

other colloids of high molecular weight. The filtrate is practically identical in composition with deproteinated blood plasma. The process of filtration is favored by an adequate rate of blood flow through the kidneys and by an adequate blood pressure in the glomerular afferent arterioles and capillaries (normally about 75 mm. Hg systolic). It is retarded by the pressure of fluid within Bowman's capsule (about 5 mm. Hg) and by the colloid osmotic pressure of the plasma proteins in the capillaries (about 30 mm. Hg). It has been estimated that the average normal rate of blood flow through both kidneys is about 1300 ml. per minute and that from 20 to 25 per cent of the plasma passes into the filtrate (125 ml. per minute). The rate will be reduced (1) by a fall in blood pressure, (2) by obstruction to blood flow through the kidney (destruction of glomeruli, obliteration of glomerular capillaries), and (3) by increased pressure in Bowman's capsule resulting from a general increase in intrarenal pressure (as in chronic passive congestion, inflammatory exudate and edema in the interstitial tissue, marked swelling of the tubular epithelium, or dilatation of the tubules from obstructions). The filtration rate can be estimated by determining the clearance of some substance (preferably inulin) which passes freely into the filtrate but is not taken up or metabolized by the tissues and is neither reabsorbed or excreted by the tubules.

The functions of the tubules are more diverse. These include: (1) Reabsorption of most of the water of the glomerular filtrate, normally about 99 per cent. Failure to reabsorb fluid normally tends to raise intracapsular pressure and diminish glomerular filtration. (2) Reabsorption in varying degree of many of the soluble constituents of the filtrate. Glucose normally is completely reabsorbed; and the maximum amount which can be reabsorbed (total passing into the glomerular filtrate less that excreted in the urine, normally 325 mg. per minute) is a measure of the *tubular absorptive mass* (glucose Tm) of the kidney. The electrolytes, like sodium, potassium, magnesium, calcium, and chlorine, are selectively reabsorbed, partly by simple diffusion and partly by a selective secretory activity of the cells, since the concentration in voided urine is usually less than in plasma. This serves a vital function in helping to maintain normal osmotic pressure and pH in the body fluids. Other substances like urea are reabsorbed to a less extent, probably by simple diffusion as the filtrate becomes concentrated, although 40 to 50 per cent of the urea passes back into the blood. On the other hand, creatinin normally is not reabsorbed at all. (3) Active excretion of certain substances, notably unnatural ingredients of the plasma like phenolsulfonphthalein and diodrast. Creatinin is also excreted, but it is very doubtful whether this is true of urea and most of the usual constituents. The maximum amount of diodrast which can be excreted per minute is a measure of the *tubular excretory mass* (diodrast Tm). (4) Synthesis and excretion of hippuric acid, and liberation of ammonia (from urea) which serves to neutralize acid bodies and conserve base.

Important as these separate functions are scientifically, the procedures for determining them have not been sufficiently simplified to be practicable for routine clinical use.

In most cases of renal disease both glomeruli and tubules are more or less involved, although in individual cases either may be primarily and predominantly damaged. The procedures which are generally used clinically as tests of renal function do not differentiate clearly between glomerular and tubular injury, although they give a fair estimate of renal function as a whole. They are designed to show: (1) inability to concentrate or dilute the urine in the normal manner; (2) reduced capacity to excrete urea and other waste products; (3) the resulting accumulation of these substances in the blood; and (4) the ability to excrete dyes and other test substances. Many clinical tests have been devised, of which only a few of major importance can be considered.

Disturbance of the concentrating power of the kidney is a most important manifestation of impaired function. It is the first to appear, it gives a good quantitative estimate of the degree of impairment, and can be demonstrated very simply, requiring no equipment except an *accurately standardized* urinometer. *Patients must be under standard conditions* when observations are made.

Dilution Test. This measures the capacity of the kidney to excrete water. The day preceding the test the patient should take a normal diet without restriction of fluid, and he should drink at least a liter of liquid during the afternoon and evening. In the morning he empties the bladder and then drinks 1200 ml. of water (within 10 or 15 minutes). He may then eat breakfast, but takes no more fluid until the test is completed. Each voiding is collected separately for four hours, at least one every half hour if possible. The volume and specific gravity of each is measured. The temperature of the specimens must be brought to that point at which the urinometer is calibrated, or a correction made. The correction factor is .001 for each 3° C. by which the temperature of the specimen deviates from the calibration temperature, to be added to the specific-gravity figure in the case of warm specimens and to be subtracted for cold ones.

A normal individual will excrete at least 600 ml. (often 1000 to 1200 ml.) during the four-hour period, and at least one of the specimens will show a specific gravity of 1.003 or less. A smaller total volume of higher specific gravity is excreted in acute nephritis, particularly in "nephrotic" cases, in chronic passive congestion of myocardial insufficiency, and in some cases of advanced chronic nephritis. In pronounced cases the total volume may be only 100 to 200 ml., and there is a tendency to fixation of the specific gravity at about 1.018 to 1.020. The test should not be given to a patient with marked edema or with cardiac failure.

Concentration Test. The performance of this test is unnecessary if the specific gravity of the morning specimen is over 1.025. As a rule this can be carried out on the same day that the dilution test is performed, just after completion of the latter. The patient eats his usual meals but takes no liquids until completion of the test. Each voiding, collected every two hours if possible, up to 10 P.M., is saved separately, and also the total night urine, preferably in two fractions. In a normal individual the specific gravity will reach 1.030 in at least one specimen, and as soon as it does, the test is completed. If it does not, additional specimens should be collected at 10 A.M. and 12 Noon the second day before fluids are resumed. A figure lower than 1.030 indicates impairment of rental function. With this there is a tendency to nocturnal polyuria and to fixation of the specific gravity which in advanced cases may be at 1.010, which is about that of protein-free plasma.

Fishberg's Concentration Test. This gives substantially the same information and is quite simple. (1) The patient takes only 200 ml. of fluid at supper the day preceding the test, and takes no fluid until the test is completed. (2) Urine passed during the night is discarded. (3) On awakening the bladder is emptied and the specimen saved. (4) The

patient remains in bed for an hour, until another specimen can be obtained. (5) He then gets up, but takes no food or fluid until a third specimen is obtained. (6) The specific gravity of each specimen is determined. Normally at least one specimen will show a specific gravity above 1.022, and usually about 1.030.

A normal individual on an average unrestricted fluid intake excretes about 1200 to 1400 ml. of urine per day. If no fluids are taken in the evening and if the period for the collection of the night specimen is begun three hours after supper, the volume of the night urine does not usually exceed 400 ml., the specific gravity is at least 1.016, and the ratio of the volume of the day urine to night urine is at least two to one, and is usually three to one. An increase in the volume of the night urine with a lowering of the specific gravity and a reduction of this ratio (nocturnal polyuria) is an early and important sign of renal insufficiency.

These tests are more sensitive than the others which are described below in detecting slight degrees of renal insufficiency, particularly in essential hypertension and the early stage of arteriolosclerosis. They can be carried out on ambulant patients anywhere. Their dependability, however, depends upon the complete coöperation of the patient, particularly as to abstaining from fluids. They are not applicable to patients with urinary obstruction with residual urine, to diabetics excreting large quantities of sugar, or to patients with diabetes insipidus.

Phenolsulphonphthalein Test (Rowntree and Geraghty). The dye is conveniently purchased already sterilized in ampules each containing slightly more than 1 ml. of a solution of its monosodium salt, of a strength of 6 mg. per ml. (1) Administer 400 to 600 ml. water. (2) After 20 minutes have the patient empty the bladder and save the urine for "backing." (3) Inject exactly 1 ml. (6 mg.) of the solution, preferably intravenously. (4) At intervals of 15 minutes, 30 minutes, and one hour instruct the patient to empty the bladder completely and save the specimens separately. If the urine volume is small, more water should be given. Catheterization is necessary if the patient cannot empty the bladder completely.

The excretion of 25 per cent or more of the dye in the 15-minute specimen indicates a normal output, and for routine purposes further specimens can be dispensed with.

For routine examination of ambulant patients intramuscular injection usually suffices. A period of 10 minutes is allowed for absorption. Specimens are collected after 40 minutes, 70 minutes, and 130 minutes.

(5) Prepare standard tubes. Make a 100 per cent standard by diluting exactly 1 ml. of dye solution to 1 liter to which 5 ml. of 10 per cent sodium hydroxide have been added. Select 15 test tubes (150 × 16 mm.) of *identical* diameter (check by adding just 10 ml. water to a series of tubes and select those in which the water rises to exactly the same height. Dry them and reserve for this purpose only). Put 10 such tubes in a rack and to each add increasing quantities of the 100 per cent dye standard from 1.5 ml. in the first to 6.0 ml. in the tenth, each tube receiving 0.5 ml. more than the preceding tube. Bring the volume of each to 10 ml. with alkalinized water. Stopper tightly and label (from 15 to 60 per cent). If stored in the dark, they will keep for several weeks, but must be renewed occasionally as the color gradually fades.

(6) Rinse each specimen of urine into a 1-liter cylinder, noting the volume. Add 5 ml. of 10 per cent sodium hydroxide and dilute with tap water to a round volume (such as 125, 200, or 500 ml.) so that the color approximates that of the standard tubes in the middle of the series. Mix, filter if not clear, and pour some into one of the matched test tubes. Into another tube put some of the urine obtained before the injection, diluted to the same extent as the specimen to be examined. Put this tube behind the standard tube for backing, and a tube of water behind the tube containing urine. Read the percentage figure on the standard tube which matches, interpolating if necessary. Multiply the figure by the volume to which the urine was diluted and divide by 1000.

The volume of the specimen does not enter into the calculation and within wide

limits the rate of excretion is independent of the volume. If the volume is small, the test loses in precision. Backing is essential, as the diluted urine is always more or less off color and if neglected the reading will be too low. Differences in the rate of absorption, the completeness with which the bladder is emptied, and other uncontrollable factors introduce unavoidable variations of at least ± 5 to 10 per cent, and attempts to secure great precision in the readings are pointless. A bizarre result necessitates a repetition of the test.

The normal figures, after intravenous injection, are: Appearance time: four to six minutes.

After ¼ hour: Minimum 25%; maximum 50%; average 35%.
After ½ hour: Minimum 40%; maximum 60%; average 55%.
After 1 hour: Minimum 50%; maximum 75%; average 65%.
After 2 hours: Minimum 55%; maximum 85%; average 70%.
After intramuscular injection: appearance time: 10 minutes.
After ½ hour: Minimum 25%; maximum 40%; average 30%.
After 1 hour: Minimum 40%; maximum 60%; average 45%.
After 2 hours: Minimum 55%; maximum 80%; average 65%.

A total output in two hours of 55 per cent or over may be regarded as within normal limits. A delayed excretion, with an increased proportion of the dye in the second-hour specimen, is abnormal even though the total output is normal. Unusually high figures with diuresis have been noted in hyperthyroidism, and in mild renal disease, and have been attributed by some to renal irritation.

The great advantages of the test are its simplicity and harmlessness. Among the disadvantages is the fact that the dye is a foreign substance which is excreted by the tubules and the capacity of the kidney to excrete it need not parallel precisely its capacity to excrete urea, although within limits it does roughly do so. It is not a sensitive indicator of lesser degrees of impaired function. In nephritis there is no notable reduction in excretion until 50 per cent of the renal function is lost, as determined by the urea clearance test. As function is further damaged, there is a progressive fall in excretion, reaching zero in the most advanced cases. Van Slyke et al. point out that with improvement in acute nephritis, a rise in dye excretion antedates an improvement in the urea clearance, and furnishes an early basis for a (relatively) favorable prognosis. The excretion may be reduced in other conditions which lower kidney function, such as chronic passive congestion and prostatic obstruction.

Urea Clearance Test (Möller, McIntosh and Van Slyke): Procedure. (1) The patient eats his usual breakfast at 8 A.M. without tea or coffee, but water as desired. (2) At 9 A.M. instruct him to empty his bladder completely and to discard the urine. Give 100 ml. water. (3) At 10 A.M. have the patient empty his bladder completely, saving the specimen, and give 100 ml. water. (4) At once secure blood for urea estimation. (5) At 11 A.M. again have the patient empty his bladder completely and save the specimen. The intervals need not be exactly one hour provided their length is precisely known. The patient should rest quietly during the test, and avoid much exertion before the test. (6) Measure the volume of each specimen and calculate the output per minute in ml. (7) Determine concentration of urea plus ammonia in each specimen. (8) Determine urea concentration in blood.

Calculation. If the volume of urine excreted is more than 2 ml. per minute calculate the maximal clearance (C_m) by the following formula.

U = urea concentration in urine.
B = urea concentration in blood.
V = volume of urine excreted per minute.
C_m = U × V/B. The average normal figure is 75, and the percentage of the normal = 1.33 × U × V/B.

If the rate of excretion is less than 2 ml. per minute, the *standard clearance* (C_s) is calculated according to the formula $C_s = U \times \sqrt{V}/B$. The average normal (with a urine volume of 1 ml. per minute) is 54, and the percentage of normal $= \frac{1.85 \times U \times \sqrt{V}}{B}$.

The chief source of gross error is incomplete emptying of the bladder. By making separate estimations on the two specimens a partial check on this is obtained, and if the results correspond fairly well, the average may be taken.

For individuals who deviate materially from the average in size the formulas must be corrected by substituting for V in each case the value of $\frac{V \times 1.73}{\text{Surface area in sq. m.}}$ (from the standard DuBois height-weight tables for surface area. Table, p. 878).

Table 79

VALUE OF $\sqrt{V}$ FOR VARYING VALUES OF V (ML. PER MINUTE)

V, ml.	$\sqrt{V}$	*V, ml.*	$\sqrt{V}$	*V, ml.*	$\sqrt{V}$
0.2	0.45	0.9	0.95	1.6	1.27
0.3	0.55	1.0	1.0	1.7	1.30
0.4	0.63	1.1	1.05	1.8	1.34
0.5	0.71	1.2	1.10	1.9	1.38
0.6	0.78	1.3	1.14	2.0	1.42
0.7	0.84	1.4	1.18	2.1	1.45
0.8	0.89	1.5	1.23	2.2	1.48

EXPLANATION. By urea clearance is meant the volume of blood which is entirely cleared of urea in one minute, or more precisely, the volume which would be so cleared if all the urea excreted in one minute were abstracted solely from one portion of blood. If the rate of urine secretion and other conditions are constant, the amount of urea excreted per minute varies directly with the concentration of urea in the blood. However, other factors than the height of the blood urea do influence urea excretion, the most important being the rate of urine excretion (volume in ml. per minute). If the rate of urine excretion is slow, the total urea excreted per minute is relatively small (even though its concentration in the urine is normal). As the volume of urine per minute rises, for a time the rate of urea excretion also rises (although not directly in proportion to the volume, but approximately in proportion to the square root of the volume). However, eventually a rate of urine excretion is reached, "the augmentation limit," about 2 ml. per minute, at which the maximum possible rate of urea excretion is obtained. Further increases in diuresis have no effect on urea output. This fact explains the necessity for the two formulas. The maximum clearance (75 ml. for normals) is constant, as long as the volume per minute exceeds 2 ml. The standard clearance is a variable, varies with the volume per minute, and the figure 54 applies (for normals) only to an excretion rate of 1 ml. per minute.

There are other factors, usually of less significance, such as the rate of blood flow through the kidney, which also affect urea excretion, and there are individual variations in normal persons which may cause variations in the clearance of at least 20 per cent above or below the average figures. Reductions of less than 25 per cent are not necessarily pathologic.

Clinical Significance of Urea Clearance Test. Within the limitations mentioned the test gives a valuable estimate of renal function. No claims are made for exact mathematical precision, and slight fluctuations are meaningless. It is questionable whether it will detect minor degrees of impairment as early as concentration tests. It does detect them much earlier than the phenolsulfonphthalein test. It is not reduced in the earlier stages of essential hypertension and arteriolar renal disease, and the fall in clearance as the disease progresses may be very gradual. Significant nitrogen retention does not appear until the clearance is below 40 per cent, and is not marked until it is below 20 per cent. Uremic symptoms appear as the clearance falls from 10 to 5 per cent. The clearance figure rises as a patient recovers from an acute attack. The urea clearance figures are regarded by many as giving the most precise and dependable measure of renal function in nephritis, obtainable with any single method.

Ratio of Urea Concentration in Urine to That in Blood. This may be substituted for calculation of the urea clearance in patients from whom it is impossible to secure complete emptying of the bladder, provided the fluid intake is so adjusted as to give a rate of urine excretion of about 1 ml. per minute. In this case the value of V becomes unity, and the simplified formula $C_s = U/B$ applies. Even moderate variations from 1 ml. alter the value of C_s (normal 54) but slightly. The results are more variable (when repeated in the same individual), and therefore less satisfactory as a routine procedure, than the urea clearance figures, but usually adequate, especially if several observations are made. The test is equally sensitive in detecting slight impairment of function. It is not valid with larger urine volumes.

Urea Concentration Test. This has been suggested as a simple procedure which avoids determining the urea in the blood. The patient is allowed no fluid after 10 P.M. In the morning the bladder is emptied, the urine being discarded, and he is given 15 Gm. of urea dissolved in 100 ml. water (flavored to disguise the nauseating taste). The total urine is collected for three one-hour periods, and measured. If the volume of the first specimen exceeds 120 ml. and that of the later ones, 100 ml., the test is useless. Otherwise the concentration of urea is determined in each. Normally it should be between 2.5 and 3 per cent in at least one specimen. A concentration below 2 per cent definitely indicates renal insufficiency. The converse is not true; a normal concentration figure can be reached with damaged kidneys if the blood urea is high enough. It is regarded as a comparatively gross test, although it usually shows renal injury well before nitrogen retention occurs.

Significance of Tests of Renal Function. The urea nitrogen and total nonprotein nitrogen are increased in the more advanced stages of renal insufficiency, in extreme cases to ten times the normal value. They are of no value in early diagnosis (see p. 770). A significant rise in creatinine is scarcely ever seen except in advanced stages of renal insufficiency, and a figure over 5 mg. %, like a N.P.N. over 100, or a urea nitrogen over 80, usually indicates impending uremia. However, in acute nephritis and in mercuric chloride poisoning the ultimate prognosis

is not necessarily unfavorable even when the N.P.N. and creatinine are very high.

In judging the functional capacity of the kidney, it is always advisable to use more than one of these procedures. The results must be interpreted in connection with the whole clinical picture.

These tests show merely the function of the kidney at the moment, but they do not, by themselves, accurately portray the anatomic condition of the kidney or furnish a basis for a dependable prognosis. A marked reduction in function may occur with circulatory failure or with severe anemia, yet normal function may be restored if the latter conditions can be remedied. An extreme reduction in function may be met with in acute nephritis, with subsequent complete recovery. On the other hand, a practically normal function may be found in the early stages of a chronic nephritis which progresses to a fatal termination within a year. The tests are relatively little affected by even extensive focal lesions of the kidney. Many patients with urethral obstruction with marked impairment speedily recover almost normal function under treatment, if there is no complicating diffuse nephritis. Successive tests which show a definite upward or downward trend are of great prognostic value. In acute nephritis Peters and Van Slyke point out that the degree to which the urea clearance is lowered at the onset is relatively unimportant, but the prognosis is very unfavorable if normal figures are not regained within four months. In chronic nephritis with a urea clearance under 20 per cent, a majority of the patients die within a year, and practically all within two years. However, patients with polycystic kidneys may live a long time with a renal function reduced nearly to zero.

Bacteriologic Examination of the Urine

Collection of Specimens. Although a catheterized specimen of urine is preferable for culture, practically it is rarely necessary in men and can often be dispensed with in women provided adequate precautions are observed in collecting the specimens and *provided cultures are made immediately on solid media.*

The bladder should be full at the time of the examination. The area about the urethral orifice should be thoroughly washed with soap and water, and then with 70 per cent alcohol (men) or with sterile salt solution or boric acid. The anterior urethra may be flushed with sterile water. The first three-quarters of the urine passed is discarded and the last portion caught directly in a sterile wide-mouth flask or bottle as it flows in an unimpeded stream from the orifice without touching the skin. The urine first passed serves adequately to flush out the urethra. The use of antiseptics is not desirable.

In the absence of infection, plates made in this way are usually sterile or show at most one or two contaminating colonies. If there is an infection of the urinary tract, the infecting organism is usually present in such predominant numbers that it is easily distinguished from a possible contaminating colony. With some experience contaminating organisms from the air can easily be recognized as such. A confirmatory culture should be made from a catheterized specimen if there are a few colonies present which may be pathogenic, or in any case if cultures are to be made in fluid media.

Before making the culture it is advisable to examine a *stained film* from the centrifuged sediment, since this may give a hint as to the type of organism present and as to the quantity of material to be spread on the plates. After centrifuging, the urine is decanted, and the tube drained by inverting it on filter paper for a few minutes. A little

sediment is removed with a capillary pipet, smeared, fixed by heat, and stained by Gram's method. If the urine does not contain albumin, to secure fixation of the film a trace of egg white or *sterile* blood serum is added to the sediment, or a few drops to the urine before centrifugation. The presence of phagocyted bacteria in pus cells is of diagnostic significance.

Acid-fast Organisms. For the demonstration of tubercle bacilli, urine may be collected over a 24-hour period, put in a cylinder, and enough sodium hydroxide added to produce a precipitate. After settling the latter is concentrated by decantation and centrifugation and digested and concentrated as described (p. 82). Some prefer to dissolve the original precipitate, after centrifuging and decanting the urine, in 12 per cent sulfuric acid. This is digested for 30 minutes at 37° C., and enough sodium hydroxide added to produce a pH of 4.5. Films are made from the precipitate after centrifugation. To avoid contamination with smegma bacilli, the same aseptic precautions required for cultures are indispensable. Smegma bacilli are not digested by antiformin. Special methods of staining to ensure decolorization of smegma bacilli are described in Chapter 4, but are not entirely dependable. If there is doubt, cultures should be made on suitable media and a guinea pig should be inoculated. Tubercle bacilli may be quite sparse in such smears, and protracted search is often required to find them. As they are apt to be in clumps, dispersion with chloroform is a valuable aid in their demonstration in films. Frequently, they are morphologically less typical than in smears from sputum.

A pyuria without bacteria easily recognized microscopically or culturally should suggest tuberculosis.

Cultures. Cultures are best made by pouring a little urine on the surface of one or two blood agar plates, and spreading with a bent glass rod. If the object of the culture is merely to demonstrate staphylococci or colon bacilli, plain agar is equally good. If typhoid or paratyphoid is suspected, SS agar or MacConkey's medium is preferable. To cultivate *Brucella abortus* an increased carbon dioxide tension is necessary.

The amount of urine to be used depends upon the estimated number of organisms present. A satisfactory procedure in the average case is to put 1 drop on the first plate and spread; and 5 drops on the second plate and spread with the same rod, without intervening sterilization. If organisms are abundant, the urine should be diluted 10 or 100 times before plating. If a sterile culture is anticipated, 1 ml. should be poured on one of the plates. To secure a growth of gonococcus the culture should be made immediately after obtaining the material from the patient.

The organisms most often met with in infections of the urinary tract are the colon bacillus, staphylococcus, streptococcus, gonococcus, proteus, typhoid and paratyphoid bacilli, brucella, and the tubercle bacillus. In most cases infection is probably derived from the blood stream. The infection may be ascending from the lower urinary passages to the kidney, particularly in cases starting after instrumentation or associated with obstruction to the urinary passages, as by a hypertrophied prostate or urethral stricture ("surgical kidney").

Pyelitis, often without characteristic symptoms, is a common cause of chronic recurring fever, particularly in pregnant women and in girls. A persistent cystitis is practically always associated with a pyelitis.

Cystitis from a colon infection gives an acid urine; that caused by proteus, an alkaline urine.

Bacterial infections of the urinary tract are associated with more or less pyuria. In general infections, particularly with staphylococci, streptococci, typhoid and paratyphoid bacilli, the organisms may appear in the urine in large numbers without necessarily causing any notable lesion in the kidney. They may disappear from the urine if the patient recovers from the infection.

No significance can be attached to the presence of bacteria, even in large numbers, in unsterile specimens of urine which have stood in the laboratory for any appreciable

time. Yeasts and molds frequently contaminate urine, especially diabetic urine, after it has been passed.

Parasites. *Trichomonas vaginalis* is a common cause of acute vaginitis in women and frequently gets into the urine in such cases.

In chylous urine filarial larvae may be found, most readily in the centrifuged sediment.

The vinegar eel may be found in the urine of women who have used vaginal douches of vinegar.

Enterobius from the vagina may be found in the urine.

The larval dibothriocephalid, *Sparganum mansoni,* has been reported three times in the urine (urethra).

The eggs of *Schistosoma haematobium* (with a terminal spine) and rarely those of *S. mansoni* (with a lateral spine) may be found in the urine.

The eggs of *Macracanthorhynchus hirudinaceus* may be recognized in the urinary sediment by their pitted appearance.

Various mites have been found in urinary sediment but are accidental contaminations.

CHAPTER 39

Examination of the Feces

The macroscopic, microscopic, and chemical examination of the feces gives important information in many diseases affecting the gastrointestinal tract. In the Tropics this is a procedure of major importance because of the great frequency and gravity of intestinal parasitism.

The *shape, size,* and *consistence* should be noted. The normal stool is formed but soft enough to be plastic. Soft or fluid stools indicate hypermotility and usually show incomplete digestion of food. Hard stools of large caliber indicate stasis, and usually atony of the colon. Small scybalous masses and stools of small caliber indicate spasm of the colon. Pencil or ribbon shaped stools suggest rectal stricture (cancer, syphilis) but may be due to spasm. In *cholera* and, occasionally, other violent diarrheas the stool is typically watery with little or no fecal material, but rather opaque from desquamated intestinal epithelium (rice-water stools). In *amebic dysentery* there are small, frequent, mucous or mucosanguineous stools, often grayish-brown and homogeneous, whereas in *bacillary dysentery* the mucopurulent stool is streaked or flecked with blood or blood-tinged mucus. Tormina and tenesmus accompany both types of dysentery.

The *motility* of the gastrointestinal tract may be checked most simply by administering a capsule containing 10 gr. of charcoal or carmine. Normally this appears within 24 to 36 hours and is entirely eliminated after 48 to 72 hours.

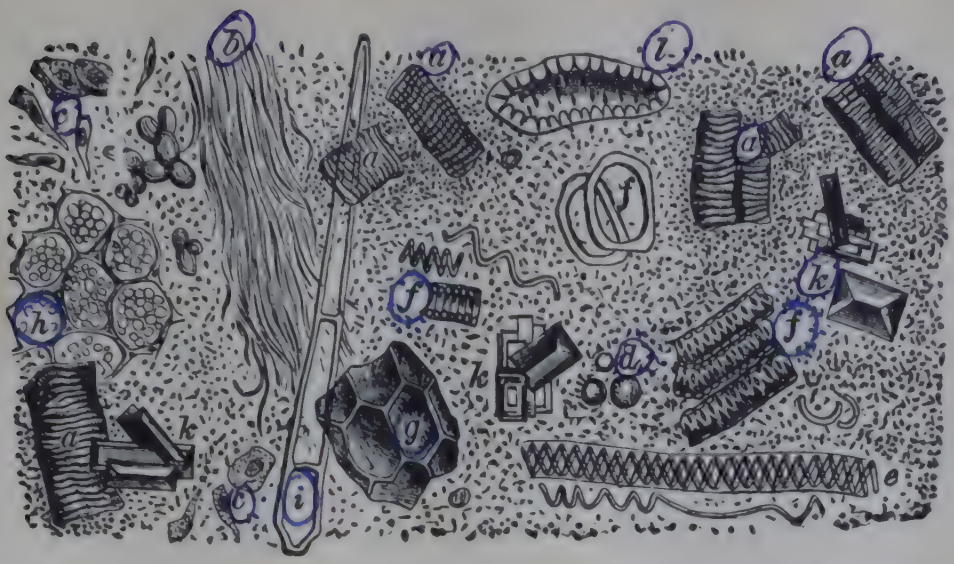
The normal brown *color* of the stool is due to urobilin (hydrobilirubin) and urobilinogen, derived from the bile pigments. Bilirubin is not normally present below the ascending colon. In the presence of diarrhea, especially in children, the feces may be green (biliverdin) or golden yellow (bilirubin). A pale, greasy, putty-like ("acholic") stool is characteristic of catarrhal jaundice and of mechanical obstruction of the common duct. A similar appearance, however, may be met with in gross disturbances of fat digestion (sprue, idiopathic steatorrhea, grave pancreatic disease). The stools are usually bulky, and soft or fluid, and fat may separate out in macroscopic drops or even in large masses ("buttery stools").

A black, tarry, viscid stool indicates bleeding from the upper gastrointestinal tract. A diffuse, red-brown or bright-red color indicates bleeding from the lower bowel. Streaks of fresh blood on the surface of a formed stool are usually due to hemorrhoids or rectal ulcerations (cancer, syphilis, ulcerative colitis).

The color varies markedly with the diet, and medication may give bizarre appearances.

Mucus is frequently present in large, translucent strands or sheets or jelly-like masses in cases of "spastic colitis" ("mucous colitis"), as a result of disturbances of innervation. This is characterized microscopically by the presence of numerous epithelial cells and often eosinophils, without pus cells. These masses have often been mistaken for tapeworms by inexperienced observers. In bacillary dysentery, ileocolitis, and intussusception the stools may consist largely of mucus in grayish, opaque masses containing many pus cells and often blood.

Frothy stools indicate faulty digestion and fermentation of carbohydrates, and are especially characteristic of sprue.



Microscopic constituents of feces. (*a*) Muscle fibers. (*b*) Connective tissue. (*c*) Epithelium. (*d*) Leukocytes. (*e*) Spiral cells. (*f, g, h, i*) Various vegetable cells. (*k*) "Triple phosphate" crystals. (*l*) Woody vegetable cells. The whole is interspersed with innumerable microörganisms of various kinds. (Von Jaksch.)

In selected cases *macroscopic examination* should be made for gall-stones, or for tapeworm segments or other intestinal parasites after a vermifuge. The entire feces (for several days if necessary) are ground up in water to form a homogeneous thin suspension, and this is strained through a sieve. The residue, suspended in water in a thin layer in a flat dish, is inspected over a dark background with the naked eye or with a hand lens. Suspected objects are removed for study. Small roundworms must be differentiated carefully from vegetable fibers and from insect larvae which may occur as a contamination. For identification see section on Parasitology.

Gall-stones can usually be recognized by their faceted surface and laminated structure. Fecal concretions ("enteroliths"), composed of masses of food residue encrusted with salts, may be confused with them. Chemical tests for cholesterin and bile pigments should be carried out. (1) A portion of the stone is crushed and extracted with a little ether. The ether is poured off; one-half volume of alcohol is added and allowed to evaporate slowly. Cholesterin if present will separate as flat, rhombic plates. A drop of strong sulfuric acid causes the appearance of a red color; a drop of Lugol's solution, a play of colors. To test for bile pigments the residue is extracted with cold dilute potassium hydroxide, and Gmelin's test performed (p. 835).

Schmidt's Test Diet. This diet should be administered for several days in selected cases in which it is desirable to study the completeness of digestion of the various types of foodstuff. Results are unsatisfactory if the diet is uncontrolled. The presence of oil or barium sulfate makes a specimen practically useless for microscopic examination. A charcoal capsule is administered at the start to indicate when the study of the feces should begin.

DIET. Breakfast, 7 A.M., bowl of oatmeal gruel (40 Gm. oatmeal, 10 Gm. butter, 200 ml. milk, 300 ml. water). Also one very soft-boiled egg (1 minute) and 50 Gm. zwieback. In the forenoon, 500 ml. of milk.

For dinner, 2 o'clock, chopped beef broiled very rare (125 Gm. with 20 Gm. butter poured over it). Also a potato purée (200 Gm. mashed potato, 50 Gm. milk, 10 Gm. butter). Also ½ liter of milk and 50 Gm. zwieback.

For supper, 7 o'clock, the same foods as for breakfast.

This detailed diet may be varied to suit circumstances as regards interchanging meals. The diet taken, however, should absolutely conform to the following requirements: (1) the taking of ¼ pound chopped beef, a portion of which should be half raw; (2) the

milk taken should amount to about a quart; (3) about 4 ounces of bread or toast and from 4 to 8 ounces of potato purée should be eaten daily.

The detailed diet contains about 110 Gm. protein, 105 Gm. fat, and 200 Gm. carbohydrates and has a fuel value of 2247 calories.

The stool is best collected in quart fruit jars and examined as soon after evacuation as possible. The wooden spatula-like tongue depressors are well adapted to handling the specimen.



(*A*) Neutral fat. (*B*) Fatty acid liberated by acetic acid. (*C*) Soaps. (*D*) Fatty acid crystals. (Courtesy, Hawk: Practical Physiological Chemistry, Philadelphia, The Blakiston Company.)

The observer must be familiar with the feces of normal individuals on this diet before attempting to interpret pathologic findings.

Macroscopic Examination. A fecal mass ½ to 1 inch in diameter is ground in a mortar; water is added gradually until the mixture has the consistency of a broth. About 0.5 ml. of this emulsion is now squeezed out between two slides and studied against a dark surface, and also when held up to the light. The normal stool gives a rather uniform, brownish, homogeneous layer. Connective-tissue remnants (indicative of gastric derangement) show as whitish fibers which swell and become translucent if 30 per cent acetic acid is added. Undigested muscle-tissue remnants appear as reddish-brown splotches, fat particles as whitish-yellow clumps. Potato remnants appear like sago grains and mash out easily like mucus. Mucus is best noted in the fecal mass before making the emulsion.

Microscopic Examination: Muscle Fibers. Muscle fibers normally appear as homogeneous, yellowish particles. Rectangular ends, distinct cross-striation, and particularly intact nuclei indicate inadequate digestion (azotorrhea).

Starch. To a drop of fecal suspension is added a drop of Lugol's solution and search is made for blue-stained starch granules.

Normally none are present except inside vegetable cells.

Fat. A bit of feces is mixed with 50 per cent alcohol, a drop of saturated solution of Sudan III in equal parts of 70 per cent alcohol and acetone is added, a coverslip is applied. *Neutral fat* appears as highly refractile droplets or yellowish flakes which stain orange to bright orange-red. Normally no appreciable amount is present. *Fatty acids* appear as flakes which stain faintly, or fine needles which tend to aggregate in clusters, do not stain, and melt on gently warming the preparation. *Soaps* appear as yellowish flakes, rounded or gnarled bodies everted like the pinna of the ear, or coarse crystals which do not melt on warming. They do not stain with Sudan III.

In feces of healthy persons the only fat elements recognizable microscopically are yellow calcium or colorless soaps. With an increased quantity of fat (steatorrhea) droplets of neutral fat appear, together with needles and splinters of fatty acids and soaps, and often an increase in the number of soap masses. In sprue from 25 to 30 per cent of the fat ingested appears in the stool, whereas the stool of pellagra, a disease possibly confused with sprue, shows only about 5 per cent, the normal figure.

Vegetable Cells. Vegetable cells and fibers, often of bizarre appearance, are always present in feces of patients on a mixed diet. Familiarity with them is necessary to avoid confusing them with ova and parasites.

Pus. Pus cells indicate an inflammatory or ulcerative lesion of the intestine, but are

sparse in amebic dysentery. They should be looked for in flecks of mucus and not in the body of the stool. Except with active diarrhea, well-preserved pus or blood cells indicate a lesion in the lower colon. Many large phagocytic cells, which may superficially resemble amebae, together with pus cells, are highly suggestive of bacillary dysentery.

CRYSTALS. Charcot-Leyden crystals, which are practically never found in the feces in bacillary dysentery, are not infrequently present in amebic dysentery. They occur also in helminthiasis and in allergic conditions, including some cases of mucous colitis. Hematoidin crystals may occur after hemorrhage. Triple phosphate and calcium oxalate crystals are often found.

INTESTINAL PROTOZOA. To demonstrate motile free-living amebae, the feces must be collected uncontaminated by urine or antiseptic solution and kept at body temperature until the examination is completed. The specimen should be examined promptly. A warm stage may be used, preferably thermostatically controlled. Overheating must be avoided. If a fluid diarrheal stool is available, a fleck of mucus should be fished out and examined (16-mm. objective). Portions of a formed stool may be emulsified in salt solution and examined, but motile organisms are not often found unless flecks of mucus can be secured. It is better to pass a rectal tube and examine mucus caught in the eye of the tube. If this is not successful a saline purge is given, and the first fluid stool is examined, some mucus being secured if possible. Positive recognition requires observation of definite and typical motility. It is helpful to add a loop of 0.5 per cent neutral red in salt solution, which vitally stains the amebae.

CYSTS. Cysts of protozoa are looked for in formed feces. The material need not be fresh or warm. A moderately thin suspension through which newsprint can just be read is made in salt solution and examined under the low-power objective. Cysts are easily located as sharply outlined, highly refractile, round structures with a diameter from one to two times that of a red cell, and can be identified with the high dry objective. They may be more easily recognized by the average observer if the drop of fecal suspension is mixed with a drop of Lugol's solution or preferably D'Antoni's solution. The cysts are tinged yellowish or brownish, and the nuclei are more distinct. Some prefer to use Wenyon's iodine-eosin solution which stains the background and most other structures pink. (See section on Amebiasis.)

D'Antoni's Solution. This solution is prepared by adding 1.5 Gm. iodine to 100 ml. of 1 per cent potassium iodide solution. The mixture is allowed to stand for four days, being shaken occasionally. It is filtered and stoppered tightly. It should be renewed after four weeks.

Iodine-eosin Solution. This consists of 2 parts of a saturated solution of eosin in physiological salt solution, 1 part of a saturated solution of iodine in physiological salt solution containing 5 per cent potassium iodide, and 2 parts of physiological salt solution.

FLAGELLATES AND BALANTIDIUM COLI. The flagellates (*Giardia, Trichomonas, Chilomastix*) and *Balantidium coli* can be found in ordinary fresh preparations.

OVA. The ova of intestinal helminths, if numerous, can be found in ordinary fresh preparations (using the low-power objective). If they are sparse, *concentration* procedures are necessary. Faust et al. (1938) recommend the following procedure. About 5 Gm. feces are rubbed thoroughly in 20 ml. warm water. Ten ml. of this are placed in a centrifuge tube, after straining through one layer of moist cheesecloth. The contents of the tube are centrifuged at high speed for one minute, the fluid is decanted, and the sediment rubbed up in water. This is centrifuged and decanted. The process is repeated several times. When the supernatant fluid is practically clear it is decanted and the sediment is suspended in a little 33 per cent zinc chloride solution. The tube is filled nearly to the brim and centrifuged at high speed for one minute. With a loop, some of the floating material is moved to a slide, a drop of iodine solution is added, and examination of the sample is made.

Other procedures especially applicable to hookworm are described in the section dealing

with hookworm. Concentration methods should supplement and not replace the usual methods of examination.

BACTERIA. Bacteria, chiefly dead, are present in enormous numbers. Except as a means of demonstrating specific pathogenic organisms, but little useful information is to be obtained from their study.

A bit of feces is suspended in 70 per cent alcohol in the concavity of a hollow ground slide. The heavy particles are allowed to sediment for a few minutes, and films are made of the suspension. These are fixed by heat or in methyl alcohol to eliminate fat, and stained by Gram's method, and for tubercle bacilli if indicated. In the normal infant Gram-positive organisms predominate (lactic acid bacilli), but in the adult most of the bacteria are Gram-negative. Gram-positive bacilli include: (1) Lactic acid bacilli, slender rods showing Gram-negative areas. They may be greatly increased in number by feeding "acidophilous milk." (2) Boas-Oppler bacilli; these may be very numerous in cancer of the stomach. (3) Gas bacilli; a marked increase has been held to indicate abnormal intestinal putrefaction. (4) Gram-positive cocci.

To demonstrate tubercle bacilli, particles of mucus or purulent material are selected if possible. They can often be found in patients with pulmonary tuberculosis who swallow the sputum, without any intestinal lesion. Concentration of tubercle bacilli in feces requires more vigorous measures than in sputum. A portion of feces the size of a pea is emulsified in 20 per cent antiformin, or 10 per cent sodium hydroxide, and heated for 30 minutes at 45° C. After centrifugation, smears are made from sediment.

CULTURES. To demonstrate organisms of the typhoid-dysentery group cultures should be made on plates of special media, such as MacConkey's agar. (See section on media.) The surface of the medium must be dry. The feces should be freshly collected in a sterile container.

It is convenient often to obtain cultures for enteric pathogens by use of the rectal swab. This eliminates the need for stool collection and all of the difficulties entailed. The beveled end of an enema tube (size F.S. 30) is cut off so that it will be about 6 inches long. A swab is prepared by twisting cotton tightly about the end of a 10-inch applicator. A drop of collodion can be used to assure security of the swab. The swab is placed in the tube so that the cotton can lie at the beveled end and both tube and swab are autoclaved. For use, a small amount of lubricating jelly is applied to the sheath and the tube is passed into the rectum for a distance of about 4 inches. The swab is exposed by pulling the sheath back about an inch and it is inoculated by rotating it around in the lumen of the gut. To remove, the swab is drawn back into the sheath and the entire tube is pulled out. The cultures are then taken and handled exactly as described for stool cultures.

For culture, a small amount of feces (the size of a pea) is rubbed up in a tube of sterile broth or salt solution. Several loops are transferred to a plate near one margin. With a sterile platinum wire or fine glass rod, the end of which is bent at a right angle to the handle about 1 inch from the tip, the material is streaked over the surface of the plate in parallel strips as wide as the bent end of the spreader. Without sterilizing the spreader, the material still adhering to it is similarly streaked successively over three additional plates. In this way a progressive dilution of the suspension is secured, the degree of dilution depending upon the diameter of the spreader. With some practice one can be certain of finding a suitable distribution of colonies on one of the plates. If such media as bismuth sulfite or SS agar are used, undiluted feces can be spread over the plates, since the growth of nearly all other organisms is inhibited. After incubation for 24 hours suspicious colonies should be fished and subcultured on Russell's medium, or Kligler's iron agar, or a preliminary macroscopic agglutination test may be made by placing a small drop of a 1 : 10 dilution of immune serum on a slide, touching the colony with the tip of a platinum needle, and rubbing this up in the drop. A positive reaction is indicated by the immediate appearance of visible clumps. Such colonies must be sub-

cultured, and the agglutination confirmed by more precise methods. Any of these media will give satisfactory results after some experience with them has been obtained, but the observer must be familiar with the appearance of the colonies of the organisms for which he is searching on the particular medium employed.

Special media for isolating cholera vibrios are described in Chapter 11.

Chemical Examination

Reaction. The reaction of the feces is normally slightly alkaline to litmus. A strongly alkaline reaction suggests faulty digestion of protein and putrefaction. An acid reaction suggests disturbed carbohydrate digestion and fermentation. The feces often become frothy on standing.

Pancreatic Ferments. Amylase and trypsin are normally present. If quantitative estimations are needed, it is better to make them on duodenal contents than on feces. Winslow's method for determination of amylase (diastase) in urine (p. 840) may be applied to feces. A filtered 10 per cent extract of feces in 0.1 per cent sodium carbonate is employed, and the results expressed as ml. of starch solution digested by 1 Gm. of feces. On a general hospital diet and after magnesium sulfate or castor oil, McClure and Pratt give normal values as 0 to 5000. Ferments are reduced or absent in acute necrosis of the pancreas, and in cases of obstruction of the pancreatic duct; they are variable in chronic pancreatitis.

Bile Pigments: Schmidt's (Qualitative) Test. A little feces is rubbed up in a saturated solution of mercuric chloride in a porcelain dish. This is observed after several hours. Urobilin, the normal pigment, gives a salmon-pink color; bilirubin, a green.

Quantitative Determination. Most of the methods for quantitative estimation of urobilin in the feces are cumbersome and require considerable experience to insure reliable results. Such estimations are often of value, however, in the study of hemolytic anemias. Normally there are marked daily fluctuations, so that the determination should be made on a specimen representing the excretion of several days. The method of Wilbur and Addis (1914) has been most frequently used, but the end point (extinction of spectroscopic bands) is notoriously inaccurate and there is no way of converting such dilution figures into mg. of urobilin excreted. These objections are met by the procedure of Elman and McMaster as slightly modified by Josephs for clinical use.

(1) Feces collected over a period of three or four days are mixed thoroughly with water and mashed through an ordinary kitchen strainer, using sufficient water so that a representative sample can be easily obtained (500 ml. to 1500 ml., according to the volume of the feces). The total volume of the suspension is measured. (2) To a small measured sample (25 ml.), three volumes of acid alcohol (1600 ml. 95 per cent alcohol, 25 ml. concentrated hydrochloric acid, and water to give a volume of 2500 ml.) are added. The mixture is shaken and allowed to stand overnight in the dark. (During this time the urobilinogen is extracted from the fecal particles and largely oxidized.) (3) After shaking, to 10 ml. of this suspension are added a drop of tincture of iodine (to complete oxidation), 0.5 Gm. zinc acetate, and 10 ml. of a saturated solution of zinc acetate in 95 per cent alcohol, freshly prepared about every three days. This is filtered until clear. (4) In one of two absolutely clean test tubes of equal caliber are placed 15 ml. standard acriflavin solution (1 : 30,000,000, freshly diluted from a stock solution containing one part in 100,000 and kept in the dark). In the other tube are placed 15 ml. diluting fluid (2000 ml. 60 per cent alcohol, 50 Gm. zinc acetate, 10 ml. concentrated hydrochloric acid, filtered until clear). To this tube are added measured amounts of the solution to be tested until the fluorescence in the two tubes matches. (For ordinary amounts of urobilin this will be in the neighborhood of 0.3 ml.) The comparison should be made against a black background with the light passing through the tubes at right angles to the line of vision. One mg. urobilin in 2000 ml. diluent matches the standard acriflavin solution when freshly prepared, saturated zinc acetate solution is used.

Calculation:

The degree of dilution of the feces in the final solution divided by 2000 gives the total excretion of urobilin in mg. for the period represented by the fecal collection. Thus, if the volume of the original fecal suspension is 1000 ml. and if 0.3 ml. of the fluorescent solution is added to 15 ml. of diluent to match the standard,

$$\frac{1000 \times 4 \times 2 \times 15}{2000 \times 0.3} = 200 \text{ mg. of urobilin.}$$

The average normal daily excretion of urobilin is roughly parallel to the amount of circulating hemoglobin; in adults, about 0.2 mg. per Gm. of hemoglobin, assuming the blood volume to be 76 ml. per kilogram. There are great individual variations. The excretion is decreased during diarrhea and also somewhat during constipation. An increase to two or three times the average may occur in a variety of conditions and usually has little clinical significance. In hemolytic anemias during the active stages the amount excreted may be 10 or 15 times the normal.

Urine, if it does not contain bile, may be treated like the filtrate in (4) above, first adding a drop of tincture of iodine. If bile is present the results are inaccurate. The amount excreted in the urine is of little importance as compared with that excreted in the feces and should not be used to estimate the severity of a hemolytic process. The ratio between the excretion in the urine and that in the feces can be used, however, as a *test of liver function.* If more than 10 per cent of the urobilin excretion occurs in the urine, it is indicative of liver damage, even in the absence of bilirubinemia.

Ordinarily there is no urobilin in the *bile,* but Elman and McMaster have shown that infection of the biliary passages may cause a conversion of bilirubin to urobilin. This suggests that the presence of urobilin in the bile may indicate an infection of the bile ducts or gall-bladder. Thus far this possibility has not been tested clinically.

Occult Blood. In all tests for occult blood, chemically clean glassware must be used. The activity of the reagents must be checked frequently, with a 1 per cent dilution of blood. A button of feces should be removed from the center of the fecal mass, to avoid surface contamination with blood from the rectum.

Gregerson Test. (1) A little feces is smeared over a glass slide. (2) A few drops of 50 per cent acetic acid, and (3) a powder containing 0.2 Gm. barium peroxide and 0.025 Gm. benzidine are added. This may be put up individually in waxed papers, or mixed in bulk, stored in dark bottles, and the quantity estimated approximately on a spatula. Result: (*a*) A deep-blue color in three seconds is a strong reaction. (*b*) A pale-blue color within 12 to 15 seconds is a moderate reaction. (*c*) A pale-blue or green color within 60 seconds is a weak reaction. The reagents are adjusted to limit the delicacy of the reaction, so as usually to avoid false-positive reactions, including those due to the constituents of an ordinary diet.

The former procedure of adding a saturated solution of benzidine in glacial acetic acid, and hydrogen peroxide directly to the feces gives many false or clinically misleading positive reactions, and only negative results are significant.

Benzidine-hydrogen Peroxide Test. (1) A moderately thin suspension of feces is made in 5 to 10 ml. water, an equal volume of ether is added, the mixture shaken well, and the ether discarded (to remove fat). (2) The residue is acidified strongly with glacial acetic acid, and (4) an equal volume of ether (which takes up the blood pigment) is added. A few drops of alcohol will facilitate the separation of the ether. (5) The reagent (equal parts of a saturated solution of benzidine in glacial acetic acid, and

hydrogen peroxide) is prepared. (6) The ether extract is stratified over the reagent, or a little of the extract and of the reagents are dropped on a filter paper. A positive reaction is indicated by the appearance within one minute of a greenish-blue, deep-blue, or bluish-black color at the line of contact. The intensity of the color and the rapidity of its appearance serve as a rough gauge of the strength of the reaction. Feeble or doubtful reactions are of no significance.

Most interfering substances are removed. The reaction is very delicate, however, and may be influenced by the diet. A positive reaction should be checked by an examination after the patient has been on a meat-free diet for three days. A reaction obtained by adding the fecal suspension directly to the reagent is much less reliable.

The orthotolidine test may be used (see Urine, p. 836). The tests using guaiac or aloin have been largely discarded, since they are much less delicate, and have no compensating advantages over benzidine.

Quantitative Determination of Fats. Cammidge's method (1914) is recommended by Beaumont and Dodds.

A. Total Fat. A few Gm. of feces are dried to constant weight over a water bath and then in a vacuum desiccator. This is ground up thoroughly in a mortar. Exactly 0.5 Gm. is transferred to a 50-ml. graduated extraction tube such as a Schmidt-Werner milk tube. Ten ml. of 30 per cent hydrochloric acid (to split the soaps) are added and the mixture is heated for 15 minutes in a boiling-water bath, the contents of the tube being mixed several times by careful rotation. The tube is cooled, filled to the mark with ether, stoppered tightly, and inverted 50 times so as to mix the contents thoroughly. It is allowed to stand upright until the ether separates. Just 20 ml. of the ether layer are pipetted into a weighed dish; the ether is evaporated and the residue dried in a vacuum desiccator and weighed. The difference (w) between this figure and the weight of the dish is the total fat in 20 ml. of ether. The volume of fluid left in the extraction tube (y) is read or measured. y — 10 ml. (the hydrochloric acid added) + 20 ml. (the ether removed), or y + 10 ml., is the volume of ether in which the fat from 0.5 Gm. of feces is dissolved. Hence:

$$\text{per cent total fat} = \frac{w \times (y + 10) \times 100}{20 \times 0.5}$$

B. Neutral Fat and Fatty Acids (without the Soaps). To determine this fraction, to a second portion of 0.5 Gm. of dried feces are added 10 ml. distilled water instead of 30 per cent hydrochloric acid. The same procedure is then followed as for total fat and the combined percentage is calculated in the same manner.

C. Fatty Acids. To determine these separately, the residue in (B) is redissolved in 20 ml. ether, a few drops of alcoholic phenolphthalein solution are added, and the resulting solution is titrated with N/10 alcoholic sodium hydroxide. The fatty acids are all estimated (arbitrarily) as stearic acid, which has an atomic weight of 284. Hence:

$$\text{per cent fatty acids} = \frac{\text{Vol. N/10 NaOH used} \times 0.0284 \times (y + 10) \times 100}{20 \times 0.5}$$

By subtracting this figure from the combined percentage in (B), the per cent of *neutral fat* is obtained.

The normal figures are given as:

Total fat	15% to 25%
Neutral fat	1% to 2%
Fatty acids	9% to 13%
Soaps	5% to 10%

In pancreatic insufficiency the total fat is greatly increased (to 60 or 80 per cent), and it is largely neutral fat. In obstructive jaundice, sprue, and other conditions in which absorption is disturbed, there is also an excess of total fat in the feces, but this is made up largely of fatty acids, since the lipase of the pancreas is not reduced in these conditions.

CHAPTER 40

Examination of the Gastric Contents

The examination of the gastric contents is a useful procedure in the study of gastric function, even though the practical value of the information so obtained is distinctly limited, and its interpretation often difficult. By this means one may obtain evidence as to (1) the motility, and (2) the secretory capacity of the stomach. In a large majority of patients with complaints referable to the stomach, the discomforts are immediately dependent upon disturbances of motility rather than of secretion. Roentgenologic examination, as a rule, is more valuable than gastric analysis in detecting motility disturbances, although it is more expensive, and requires considerable experience for accurate interpretation. Gastric analysis, however, often gives valuable confirmatory information, and is the only direct method of determining secretory capacity.

The points of chief importance to be determined by examination of the stomach contents are: (1) Evidence of stasis; an abnormal volume of fluid and food retention 12 hours after a stasis meal (important). (2) The time required for the stomach to empty itself after a standard test meal, or the volume remaining after a definite time interval. (3) The capacity to secrete hydrochloric acid and enzymes (important). (4) The degree of acidity and the quantity of enzymes formed after a standard stimulus. (This is less important. So many variable factors influence acid secretion that only gross variations from normal have any significance.) (5) Presence of abnormal constituents (lactic acid, bacteria, pus, blood, etc.). (Important in selected cases.) (6) The degree of digestion of the food (of minor importance).

There is as yet no general agreement as to the best procedure for such an examination. Opinions differ as to the best meal to give, as to the type of tube to use, and as to the time at which the contents should be removed.

Examination of *vomitus* is much less satisfactory than that of material obtained through a tube because it is always mixed with saliva and mucus from the mouth, and often with regurgitated duodenal contents, which dilute the gastric contents and directly neutralize some of the acid. However, it should always be examined when available, if passage of a tube is not advisable. Passage of a tube is absolutely contraindicated in the presence of an aneurysm of the aorta, esophageal varices (cirrhosis), obstruction of the esophagus (cancer, stricture), and usually with an esophageal diverticulum, a bleeding ulcer, and in myocardial insufficiency, extreme hypertension, or coronary disease (angina).

Passage of the Tube. The large, semi-rigid tubes formerly used cause so much retching and discomfort to the patient that they are now rarely employed for test meals. An additional disadvantage is that the stomach cannot be emptied so completely with them as with a small tube. They must be used for gastric lavage in case of poison-

ing, or in case the lumen of the small tube becomes plugged with mucus or coarse food particles.

The Rehfuss tube or one of its modifications is used extensively in this country. The Ryle tube (with a closed tip weighted with lead) is much used in Great Britain. The patient (if able to do so) should sit upright in a chair with the head thrust slightly forward. The metal bulb is dropped behind the tongue, and the patient is instructed to swallow it, and to continue to swallow, as he sucks the tube into the mouth, until the 50-cm. mark reaches the teeth. It is often necessary to give a small (measured) amount of water with the tube.

We prefer to use a catheter type of duodenal tube with lateral openings, such as the Levin tube (No. 16), or Sawyer tube, or, in cases offering difficulty, a Jutte tube, which is passed by means of a blunt-tipped wire stylet, and requires no active coöperation from the patient. The tube is moistened and chilled in ice water. The patient is instructed to keep the mouth open and to breathe rapidly and deeply through the mouth to control retching. The tip of the tube is pushed into the pharynx, and the patient is told to swallow once or twice to direct the tip into the esophagus. It can then usually be passed on into the stomach without further swallowing. If necessary the throat can be sprayed with 2 per cent cocaine solution. If one nasal passage is free from obstruction, it may be cocainized and a No. 12 or 14 Levin tube passed with a minimum of discomfort, but this is not recommended as a routine procedure. The patient is instructed to expectorate (and not swallow) any saliva or mucus that accumulates in the mouth.

Test Meal. A *stasis meal* should be given 12 hours before the test if stasis is suspected. It may consist of rice pudding containing raisins, or a charcoal powder may be administered. The test meal should be given in the morning, with the stomach "empty." The tube should be passed and the stomach completely emptied before the test meal is given. To do this effectively the aspiration should be carried out with the patient recumbent, prone, and in both lateral positions. The normal *fasting stomach* contains from 20 to 100 ml. (occasionally 150 ml.) of fluid (average 50 ml.). A larger volume of fluid indicates a gross disturbance of motility. The presence of appreciable amounts of food residue nearly always means organic pyloric obstruction (usually due to ulcer or cancer). The contents should be saved for the usual examinations later. Free hydrochloric acid is normally present (circa 20), but is often absent in patients who show normal amounts of acid after a test meal.

The Ewald breakfast is still widely used: 2 thin slices of dry bread or toast (without butter) (35 Gm.), well masticated, and 2 cups (250 ml.) of clear tea (or preferably water, either hot or cold). In place of the bread, one may give a shredded wheat biscuit, or eight arrowroot biscuits (which are free from lactic acid). This may be eaten with the small tube in place after the stomach has been emptied if the patient prefers this, rather than removal and re-introduction of the tube. The Boas meal (1 pint of strained oatmeal gruel) is also widely used.

Many prefer an alcohol meal, 100 ml. 7 per cent alcohol, injected through the tube. Some give 300 ml. 5 per cent alcohol. The chief advantage is that the stomach contents are "cleaner," and less likely to obstruct the tube than if food is administered. All are about equally satisfactory as stimulants of gastric secretion. The alcohol meal leaves the stomach more rapidly than the Ewald meal. Bergeim, Rehfuss, and Hawk showed that plain water is an efficient stimulus.

Removal of Gastric Contents. Many still follow the original method of Ewald, removing the *entire* contents of the stomach one hour after the patient started to eat the meal.

Others prefer *fractional removal*. A small tube is passed 30 minutes after the meal, and is left in place. A small sample (5 ml. to 10 ml.) is withdrawn at 15-minute intervals until the stomach is empty, or until 8 to 10 samples have been obtained. The stomach is emptied completely at the last aspiration. The specimens are examined individually. The stomach, however, does not mix its contents thoroughly. If a series of

samples are withdrawn in rapid succession, either with or without altering the position of the tube, the various fractions often show marked differences in the degree of acidity present. The results of a fractional removal may, therefore, be misleading, unless the entire stomach contents are thoroughly mixed by repeated aspiration and reinjection prior to each removal. If this precaution is taken the method gives more complete and precise information as to gastric function than does a single aspiration, and often shows free hydrochloric acid in patients in whom it was not present in the single (one-hour) specimen. One may question, however, whether the practical value to the patient of the additional information obtained adequately repays the extra time and discomfort involved, except in selected cases. Bloomfield and Keefer (1926) have devised an elaborate procedure for estimating also the rate of secretion and discharge from the stomach.

Histamine Injection. Histamine given hypodermically furnishes the strongest known stimulus to gastric secretion, and is the crucial test in differentiating between "true" and "apparent" achlorhydria. Its administration is not to be recommended as a routine procedure, but it should be used, as a rule, in all persons showing no free hydrochloric acid by other methods, particularly in patients suspected of pernicious anemia or idiopathic hypochromic anemia. A small tube is passed (fasting), and the stomach is emptied, the tube being left in place. Instead, the test may be given at the conclusion of one of the meals previously described, if no free hydrochloric acid has been secreted. Three-tenths mg. histamine (as histamine or ergamine phosphate) is injected hypodermically. Nothing is given by mouth. The stomach is emptied after 15 minutes, and again after 30 minutes. Occasionally a third specimen at 45 minutes is desirable. The dose of histamine originally advised (0.01 mg. per kilogram) gives a somewhat greater response, but often causes disagreeable reactions, and is not without danger.

Bockus and others have shown that about 50 per cent of the patients who show no free hydrochloric acid after an ordinary Ewald meal will show it after a fractional analysis, and an additional 25 per cent will show it after histamine injection. The normal range of acidity is from 40 to 140, usually between 90 and 125. Patients with benign gastric ulcer practically always show free hydrochloric acid of 30 or higher. The normal volume in the 15-minute period varies from 35 ml. to 150 ml.

Examination of Specimens

Gross Appearance. 1. The *volume* is measured and recorded. Delayed emptying is indicated by a volume of more than 150 ml. in the fasting stomach, of more than 100 ml. one hour after an Ewald meal, or by the presence of food and appreciable amounts of fluid more than two and one-half hours after such a meal fractionally removed.

2. The *color* is noted. A yellowish or greenish tint indicates regurgitated bile. Small amounts are normal. Streaks of fresh blood are usually due to trauma from the tube. A diffuse reddish or brownish color suggests gastric bleeding. The "coffee grounds" sediment composed of blood altered by prolonged stasis suggests cancer.

3. A foul or rancid *odor* in the fasting contents suggests cancer.

4. *Mucus* is normally present in small amounts. Excessive amounts most often come from swallowed sputum or nasopharyngeal secretion. This is often frothy, floating on the surface of the fluid in tenacious masses, and contains squamous epithelium, pus cells, and other constituents of sputum. Mucus may be secreted by the stomach in large amounts, particularly in chronic gastritis. It usually contains swollen gastric (columnar) epithelial cells and a few pus cells.

5. Search is made for gross *food residue* in the fasting contents. The amount of food residue and the degree of its digestion after a meal are estimated roughly.

Microscopic Examination. Microscopic examination of the residue from the fasting stomach is important, and must be made immediately. Examination of the sediment after a test meal rarely yields any useful information. Look for:

1. *Food particles,* particularly starch granules (test with iodine).

2. *Blood or pus cells.* A few pus cells have no significance. Pus cells in appreciable quantity are nearly always swallowed (pyorrhea alveolaris, chronic sinusitis or nasopharyngitis). Pus of local origin is seen infrequently in ulcerating cancer, and rarely in acute suppurative infections of the stomach wall.

3. *Tumor tissue.* Small fragments of tumor tissue may be found in very rare instances. Recognition is difficult.

4. *Boas-Oppler bacilli.* These are very large (1 × 5 to 10μ), Gram-positive, non-spore-bearing organisms, growing in long chains and forming large masses. They are lactic-acid producers and can be cultivated aerobically on media rich in milk or blood. They



Microscopic constituents of the gastric contents. (*A*) Starch cells. (*B*) Yeast cells. (*C*) Boas-Oppler bacilli. (*D*) Staphylococci. (*E*) Streptococci. (*F*) Sarcinae. (*G*) Muscle fiber. (*H*) Mucus. (*I*) Red blood cells. (*J*) Leukocytes. (*K*) Snail-like mucus formations. (*L*) Squamous epithelial cells. (*M*) Cellulose. (Courtesy, Hawk: Practical Physiological Chemistry, Philadelphia, The Blakiston Company.)

occur when stasis exists and hydrochloric acid is absent, chiefly in cancer. They are significant only when present in large numbers.

5. *Sarcinae.* In large numbers these indicate stasis in the presence of hydrochloric acid, and are seen most often in gastric ulcer with pyloric obstruction.

6. *Parasites.* These are rarely found. Flagellates have been found occasionally in cases of anacidity, as in some cases of early cancer.

Chemical Examination

Normal stomach juice contains not only free (ionizable) hydrochloric acid, but also organic acids and all the diffusible constituents of the blood plasma, including salts and some protein which act as buffers and interfere to some extent with obtaining precise titrations. There is no reagent or indicator which gives a specific reaction with free hydrochloric acid as such. The color shown by the indicator depends upon the pH of

the solution. As such a mixture is neutralized by a strong base, appreciable amounts of ionizable hydrochloric acid disappear when the pH has risen to about 4.0. Töpfer's reagent is used as an indicator of free hydrochloric acid because it shows a fairly sharp change in color when the pH has been raised to about this point.

Free Hydrochloric Acid: QUALITATIVE DETERMINATION. Filter some of the gastric contents through cheesecloth or soft filter paper, and put a measured amount (10 ml. if available) in a porcelain dish or small flask over a white background. Add 3 drops of Töpfer's reagent. If free acid is present, the fluid turns a bright cherry-red color.

TÖPFER'S REAGENT. Five-tenths Gm. dimethyl-amino-azo-benzol in 100 ml. 95 per cent alcohol.

BOAS' TEST. If the color obtained with Töpfer's reagent is indeterminate, Boas' test may be used in confirmation. In a porcelain dish put a few drops of Boas' reagent and evaporate to dryness (do not scorch). Add a drop of stomach contents and heat gently. The appearance of a rose-red color which fades on cooling indicates the presence of free mineral acid. (The acid hydrolyzes the sugar, liberating levulose, which reacts with the resorcinol to produce the color—Seliwanoff's reaction.)

BOAS' REAGENT. Resorcinol, 5 Gm.; cane sugar, 3 Gm.; 50 per cent alcohol, 100 ml.

QUANTITATIVE ESTIMATION OF FREE HYDROCHLORIC ACID. From a buret add N/10 sodium hydroxide (N/100 if only 1 or 2 ml. of filtrate are available) until the color changes to a dull salmon-pink (Michaelis). Many prefer to titrate until a canary-yellow color is obtained, as the color change is sharper. The results are slightly too high, but this is of no practical significance. Observe the volume of alkali added.

Calculation:

$$\frac{\text{No. of ml. of N/10 NaOH used} \times 100}{\text{Volume of sample titrated}} = \text{Free HCl, expressed as ml. of N/10 acid per}$$

100 ml. of gastric juice. This may be abbreviated to N/10 acid %, or referred to simply as "points." It seems undesirable to use the term "degrees," which has been used to indicate units of an entirely different sort.

To convert this figure into absolute per cent of acid, multiply by 0.00365.

Total Acid (Quantitative Estimation). To the same sample, after completing the preceding titration, add 3 drops of a 0.5 per cent solution of phenolphthalein in 95 per cent alcohol, and add N/10 sodium hydroxide from a buret until a distinct pink color is obtained which persists after stirring for several seconds. Observe the total amount of alkali added in both titrations.

Calculation:

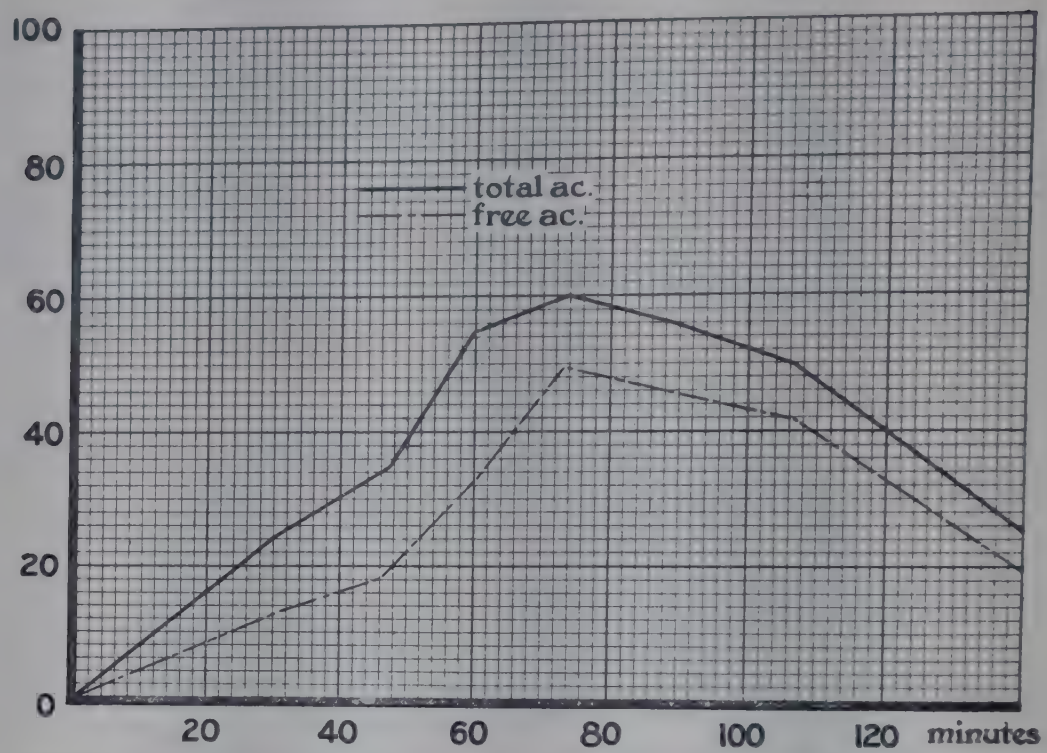
$$\frac{\text{Total No. ml. N/10 NaOH used} \times 100}{\text{Volume of sample analyzed}} = \text{total acidity in N/10 acid \%.}$$

The normal figures for an Ewald meal are from 25 to 50 free hydrochloric acid, and from 50 to 80 (or even to 100) total acid. The average normal figures for a fractional analysis are shown in the chart from Hawk.

The difference between the "total acid" and the free hydrochloric acid is commonly spoken of as the *"combined acid."* It includes the hydrochloric acid combined with protein, the acid salts, and organic acids if present. Separate determination of these fractions is of no clinical value.

Hydrogen-ion Concentration. The hydrogen-ion concentration may be determined by the method described (p. 926) or more simply by the use of test papers (Töpfer's reagent and thymol blue), comparing the color obtained with standard charts. The additional information so obtained is rarely of much practical value.

Lactic Acid: KELLING'S TEST. To a test tube of distilled water add 3 drops of 10 per cent ferric chloride solution (fresh and light in color), and mix. Pour half into a similar tube as a control. Into one tube put a small amount of filtered gastric juice.



Acidity curves of normal human stomach. (Courtesy, Hawk: Practical Physiological Chemistry, Philadelphia, The Blakiston Company.)

Inspect by looking down into the tubes against a white background. A distinct canary-yellow color indicates the presence of lactic acid. Slight traces are of no significance.

Lactic acid (occasionally with acetic and butyric acid) occurs only when stasis exists in the absence of hydrochloric acid, and strongly suggests cancer. It should be tested for in all cases with achlorhydria or marked subacidity.

Pepsin. Pepsin is always present if hydrochloric acid is secreted, and may be present in cases showing a complete achlorhydria. Clinically its estimation is of practical value only in such cases, in which it is desired to determine complete loss of gastric function (*achylia gastrica*).

METT'S METHOD. Mett's method is the most practicable. Into each of 2 test tubes put 2 (better 4) Mett tubes. To one tube add 15 ml. N/20 hydrochloric acid. To the other tube add 1 ml. filtered gastric juice diluted with 15 volumes of N/20 hydrochloric acid. Incubate 24 hours at 37° C. Note whether the ends of the column of coagulated albumin in tubes in the diluted gastric juice have been digested, and if so measure accurately (in mm.) the length of the digested portion in each of the 4 (or 8) open ends, and take the average. The control tubes should show no digestion.

To express quantitatively in Mett units, square this average figure and multiply by 16 (the dilution). Normal figures are 2 to 4 mm. digested, or 64 to 256 units. Such figures are only approximations. Different specimens of albumin may show considerable differences in the amount digested when tested in the same specimen of gastric juice. If little or no digestion has occurred, the presence of small amounts of pepsin may be checked by repeating the test with higher concentrations of gastric juice.

METT'S TUBES. To prepare the tubes, select capillary glass tubing with an internal diameter of 1 to 2 mm. Clean, break into 6- or 8-inch lengths, and fill (by suction or immersion) with clear, filtered egg albumen, free from air bubbles. Plug the ends with bread crumbs, and coagulate by immersion in a water bath at 85° C., leaving them in the water until the bath cools. Seal the ends with sealing wax, and store under water in the icebox. When needed, break the tubes squarely into sections about 2 cm. long, and discard any portions the lumen of which is not completely filled, or which contain air bubbles.

Blood. Blood should be tested for by the benzidine or orthotolidine method (see Feces). Traces are of no significance (trauma of the tube). Bleeding in ulcer and cancer can be followed more conveniently by examination of the feces.

Bacteriologic Examination. Bacteriologic examination of stomach contents rarely yields information of practical value except to demonstrate tubercle bacilli in swallowed sputum. Normally the contents are sterile. If achlorhydria is present, the usual mouth flora are obtained. Tubercle bacilli can frequently be demonstrated in swallowed sputum from patients who fail to expectorate. The stomach contents should be examined for tubercle bacilli in every suspected case of tuberculosis in which the organisms are not readily found in sputum. The fasting stomach contents should be aspirated through a tube as described, and the mucus and sediment examined by means of stained films and cultures, as in the examination of sputum, preferably after concentration. Culture or guinea-pig inoculation is preferable to stained film, since nonpathogenic acid-fast bacilli occur about five times as frequently in stomach contents as in sputum.

Interpretation of Examination

Interpretation of the results of these examinations requires much judgment and discrimination. In an untrained patient the results of the first examination are often misleading. Unexpected or bizarre results should always be checked. In practically all cases a definite diagnosis can be reached only in conjunction with the history and other findings.

In a majority of patients with gastric complaints (other than those due to gross dietary indiscretions) the symptoms are due to *functional disorders* brought about by disturbances of innervation, and not by organic disease in the stomach itself. Both motility and secretion are under nervous control. These disturbances may arise as a "reflex" from organic disease elsewhere, such as chronic appendicitis, cholecystitis, ureteral stricture, or pelvic disorders. In many cases they are simply a result of abnormal nervous tension, or of purely psychoneurotic disturbances. There is no finding characteristic of purely functional disorders. Pylorospasm and hyperacidity are common, but atony and subacidity occur. A great variation in the results of successive examinations is a suggestive finding.

Delayed motility may be due to (1) organic pyloric obstruction (cancer, ulcer with stricture, adhesions); (2) pylorospasm (functional or reflex disturbances of innervation, ulcer); (3) atony (most often seen in undernourished, asthenic individuals with visceroptosis, or as a purely functional disturbance).

Rapid emptying of the stomach is met with chiefly in (1) achlorhydria; (2) functional disturbances; (3) duodenal ulcer; (4) rarely in cancer infiltrating but not obstructing the pylorus.

Hyperacidity. It has been shown by Boldyreff and others that normally pure gastric juice contains 0.4 to 0.5 per cent hydrochloric acid, and that the usual concentration of 0.15 to 0.2 per cent is the result of dilution and neutralization by ingested food and fluid and by regurgitated duodenal contents. Hyperacidity is often associated with pylorospasm, which retards or prevents such regurgitation. It further causes a prolonged retention of food residue which stimulates a protracted and perhaps accelerated rate of acid secretion. There is often a progressive rise in acidity throughout the fractional analysis. Hyperacidity is met

with in many cases of gastric ulcer and most cases of duodenal ulcer, but is a common result of functional or reflex disturbances.

Achlorhydria. Achlorhydria of a temporary character occurs as a functional disturbance, and often in fevers and in debilitated individuals. True achlorhydria (or a marked subacidity) occurs (1) as a constitutional defect (often symptomless); (2) in many elderly individuals (a senile degenerative process); (3) in chronic gastritis (due to alcohol, faulty habits of eating, chronic focal or systemic infections, etc.); (4) in pernicious anemia and idiopathic hypochromic anemia; (5) in some cases of chronic gall-bladder disease; (6) in most cases of cancer except those arising in a chronic ulcer. The achlorhydria often antedates the cancer, and is not necessarily a direct result of the cancer.

Achylia Gastrica. Achylia gastrica (with loss of ferments as well as acid) occurs in most cases of pernicious anemia, and in a smaller proportion of the other conditions listed above.

Gastric Ulcer. In gastric ulcer there may be a normal or an increased acidity (rarely a subacidity), often pylorospasm and delayed emptying, and the intermittent presence of blood. These findings change but little if cancer develops in the ulcer. A large volume of fasting contents and a total acidity over 70 suggest a chronic ulcer with obstruction.

Carcinoma. In an advanced stage, carcinoma may often be recognized by demonstrating achylia or achlorhydria, evidences of obstruction (large volume of fluid with 12-hour food-retention, lactic acid, Boas-Oppler bacilli), and blood (present in a majority of the cases). In the early stages a diagnosis can rarely be made by gastric analysis alone.

CHAPTER 41

Examination of the Duodenal Contents and the Bile

These examinations are so tedious and so uncomfortable to the patient and the information they yield is usually so meager that they are indicated only in selected cases. In these few cases they may be of great value. Practically, examination of the duodenal contents is useful chiefly as a means of measuring the pancreatic ferments in suspected pancreatic disease. Examination of the bile gives positive information in some cases of cholecystitis and cholelithiasis, but in many cases no definite conclusions can be drawn. Much experience and critical judgment are required for the interpretation of the findings, and these must always be considered in conjunction with all the information obtainable by other methods of examination.

Procedure. The examination is made in the morning with the patient fasting. A duodenal tube is passed to the 55-cm. mark, as previously described, and the stomach is completely emptied by aspiration and lavage. The patient then lies on the right side with the hips elevated and the tube held loosely in the mouth. By taking a few swallows of water and sucking in about 1 inch of the tube every five minutes (up to about the 75-cm. mark), the latter will usually enter the duodenum within 30 minutes to an hour. Pylorospasm may be overcome by injecting hot water through the tube, or by giving 1 mg. of atropine sulfate. Attempts to hurry the passage of the tube frequently result in its coiling up in the stomach. It must then be withdrawn to the 55-cm. mark, and the process repeated more gradually. The tube should be filled with water, and the drainage obtained by siphonage collected in small flasks or bottles, using a new bottle whenever the appearance or color of the fluid changes. The arrival of the tube in the duodenum can usually be recognized by the change in the character of the fluid. If in doubt, this can be verified by the "duodenal tug," a prompt collapse of the tube when suction is exerted; and by the failure to recover promptly, by aspiration, water or colored fluid which is swallowed.

Duodenal Contents. Normal duodenal contents consist of a thick, viscid, ropy fluid, clear or faintly opalescent, pearly-gray or light-yellow (bile), and alkaline to litmus unless mixed with gastric juice. It is a mixture in varying proportions, of duodenal secretion, pancreatic juice, bile, and gastric juice. Little or no useful information is obtained by titration or microscopic examination.

Pancreatic Ferments. These may be measured by the method of McClure et al (1921). The following simpler methods suffice for all practical purposes. Only gross variations from normal are significant. Estimations in duodenal contents are more reliable than in feces. The fluid should first be made 8 degrees alkaline to phenolphthalein with sodium hydroxide.

Trypsin. We employ Gross' method in the following manner: In a series of 6 small test tubes, place 1.0, 0.5, 0.25, 0.1, 0.05 and 0.01 ml. alkalinized duodenal contents. To each add 2.5 ml. 0.1 per cent casein in 0.1 per cent Na_2CO_3, and mix. Add a seventh tube for control, containing only casein solution. Digest in water bath at 38° C. for 15

minutes. Add a few milliliters cold water; then acidify each tube with dilute acetic acid, and mix. Note least amount of duodenal contents producing complete digestion as indicated by transparency of the mixture, and report as number of milliliters of casein solution that 1 ml. duodenal contents will digest. If this is the tube containing 1 ml. duodenal contents, report would be 2.5; if one containing 0.1 ml., 25, etc. We consider the normal to be 25 to 50.

Amylase (Amylopsin). We apply Wohlgemuth's method in the same manner as in the previous test, using 1 per cent soluble-starch solution instead of casein, and digesting for 30 minutes. Test for starch with a few drops N/20 iodine in each tube. The tube with the least amount of duodenal contents, showing no blue color when mixed, is determined, and the result calculated as for trypsin. We consider the normal to be 2.5 to 5.0.

Lipase (Steapsin). Place 1 ml. alkalinized duodenal contents in each of 2 large test tubes. Boil contents of one, and cool. To each tube then add 1 ml. ethyl butyrate, 10 ml. water, and 1 ml. toluene. Shake well, and incubate at 37° C. for 24 hours, shaking several times in the interval. Then titrate acidity, using N/10 sodium hydroxide and phenolphthalein. The difference (in milliliters) between the results of the two titrations represents the lipase. We consider the normal to be 0.2 to 2.0.

Parasites. Parasites have been found in rare instances: *Giardia lamblia, Strongyloides stercoralis, Endamoeba histolytica,* two-celled hookworm ova.

Bacteriologic Examination. Cultures are of practical value only when the contents contain infected bile. Normally they are sterile. If achlorhydria exists, mouth organisms may be obtained. Regurgitation of colon bacilli and other intestinal organisms is rare.

Bile. After sufficient duodenal contents have been collected for examination, slowly inject 50 ml. of hot 25 per cent solution of magnesium sulfate through the tube. (If purging is contraindicated, hot bouillon or hot water usually suffice. Dilute hydrochloric acid is an efficient stimulus, but it precipitates the fats and makes the specimens useless for examination.) Siphon (or if necessary aspirate) out as much of the solution as possible and collect the ensuing drainage in small bottles. To get satisfactory specimens it is necessary to watch the drainage constantly, and to change the container with each change in the appearance of fluid. As a rule, after from 5 to 30 minutes bile appears. If the duodenal contents were colorless, the first few ml. are usually bright light-yellow in color (Lyon's "A bile," which he believed comes from the ducts). This is quickly followed by darker bile, "B bile," which undoubtedly comes from the gall-bladder, but is inevitably diluted more or less with duodenal secretion and pancreatic juice. This fraction usually comes out rapidly in spurts, until from 5 to 50 ml. (or even 100 ml.) have been obtained. Normally this is deep golden-yellow in color, viscid and ropy, clear, and alkaline to litmus. This is often interrupted by spurts of cloudy, opaque, yellow ("egg-yolk") fluid, owing to an admixture of gastric juice. (If this is allowed to mix with the clear fractions, the specimen will be ruined.)

If no "B bile" is obtained within 30 minutes, a second and even a third injection should be tried. Flow may sometimes be stimulated by abdominal massage, or by having the patient walk about.

The B bile is followed by the slow, protracted drainage of a lemon-yellow fluid, "C bile," which Lyon believed comes directly from the liver.

Failure to obtain B bile may be due to: (1) Premature discharge from the gall-bladder, before the tube is in place. (2) Spasm of the sphincter muscle (probably). (3) Obstruction of the cystic duct by stone or adhesions. (4) Failure of the gall-bladder to fill and contract in a normal manner, usually due to cholecystitis. Total absence of pigments indicates complete obstruction of the common duct.

The *color* of the B bile may be abnormally dark: a golden-brown to a greenish-black. Such fluids darken rapidly and turn greenish on standing. This indicates stasis in the gall-bladder, a condition which predisposes to stone formation. The fluid may be *cloudy* and

contain considerable *mucus* in cases of cholecystitis. (Exclude acid from the gastric juice, and swallowed sputum.)

Microscopic examination of the centrifuged sediment must be made immediately. Look for: (1) Pus cells which are bile-stained and scattered through the specimen in fair numbers point strongly to a cholecystitis. Unstained, "cleared" pus cells, and masses of mucus containing many pus cells are usually from the mouth. (2) Bile-stained epithelial cells and granular, cellular detritus suggest chronic cholecystitis. (3) Cholesterin crystals, as translucent or opaque, impure, flat, rhombic plates, often in irregular masses. (4) Calcium bilirubinate, in the form of light-brown granules or dark red-brown or blackish precipitate. Large amounts of both these substances are highly suggestive (90 per cent) of gall-stones. Either or both in small amounts have no significance. (5) Rarely, microscopic gall-stones ("bile sand"), as small, concentrically laminated concretions. Fatty acid and soap crystals often separate out on standing, but have no significance.

Negative findings are not conclusive.

Cultures of B bile from cases with active cholecystitis not infrequently reveal the causative organism, but the results of cultures must be interpreted with caution, particularly if achlorhydria is present. We spread the material on plain agar and blood agar plates, using 1 loop of material in one series, and 1 ml. in a second series. Cultures from the fasting stomach (and from the duodenal contents, if free from bile) are useful as controls. Perfect asepsis is impossible, and a few colonies must be disregarded, although normal bile usually yields entirely sterile cultures. A fairly abundant growth of a possible pathogen in relatively pure culture is usually significant, particularly if the control cultures do not show them. Colon bacilli are most often obtained, occasionally staphylococci, or streptococci. Typhoid bacilli can usually be obtained in culture from carriers, more easily and more regularly than from the feces.

CHAPTER 42

Tests of Liver Function

The liver performs, or participates in the performance of, many different and apparently unrelated functions of vital importance. Among others it brings about (1) deamination of amino acids and synthesis of urea; (2) conversion of glucose, fructose, galactose, and lactic acid into glycogen, and storage of glycogen; and (3) mobilization and delivery of glycogen as glucose to the blood, so as to maintain approximately a constant blood-sugar level; (4) it takes up bilirubin (and urobilinogen) from the plasma and excretes it in the bile; (5) it synthesizes and excretes bile salts; (6) it excretes cholesterol; (7) it detoxifies many substances, both extraneous poisons (e.g., strychnine, cinchophen) and protein cleavage products of digestion; (8) it excretes certain dyes; (9) in most mammals except man it oxidizes uric acid; (10) it forms fibrinogen and probably other plasma proteins; and (11) through its Kupffer cells it participates in all the manifold functions of the reticulo-endothelial system, including the removal of bacteria and other particulate matter from the blood and the synthesis of bilirubin from hemoglobin.

Many different tests of liver function have been devised, based on these activities, but none has proved satisfactory as a means of detecting early or slight impairment of function. Many have been abandoned because experience has shown that they are unreliable or too insensitive to be of any practical value.

It is obvious that no single test can measure adequately the capacity of an organ with so many different functions. These functions in diseases of the liver are not depressed uniformly, nor is there any constant relation in the degree to which the different activities are depressed. It is not possible to associate any type of liver lesion with any one disturbance of function. Some of them are so much influenced by the activities of other organs that they are unreliable as an indication of liver injury (e.g., the storage and mobilization of glucose is influenced by insulin and epinephrin). Furthermore, the reserve capacity of the liver is so great, from 75 to 95 per cent for most of its activities, according to animal experiments, that its function is measurably altered only if the injury is diffuse and severe, and some functions (e.g., synthesis of urea) are maintained until destruction is practically complete. Focal lesions, even if extensive, rarely affect the usual tests appreciably, and even in cases of extensive cirrhosis compensatory hyperplasia may be adequate to maintain normal function according to the tests.

It follows that one should not depend upon any single functional test, but if possible several should be used. A normal result with any or all the tests does not exclude disease of the liver. On the other hand, if errors in technic can be excluded, a definite impairment of function revealed by any one reliable test is positive evidence of disease of the liver. Although in a very general way the more severe the liver injury is, the more

impairment the functional tests will show, there is no close correlation between the degree of impairment and the gravity of the disease. However, a progressive improvement or deterioration of function, as shown by repeated tests on the same individual, has definite prognostic value.

Disturbances of Bile Excretion

These are characterized by *hyperbilirubinemia.* Bilirubin is derived largely if not entirely from the hemoglobin of disintegrated red blood cells (and possibly from muscle hemoglobin). The process is discussed in the section on Hemolytic Anemias (p. 424). Hyperbilirubinemia is met with clinically: (1) in mechanical obstruction of the bile ducts; (2) in accelerated blood destruction; (3) in diffuse liver injury. In the latter group it is believed that two factors play a part: (1) the damaged liver is unable to remove bilirubin from the plasma as fast as it is formed, even though there is no pathologic acceleration of hemolysis; and (2) if damage is intense and necrosis of liver cells extensive, the bile canaliculi probably become blocked, and the bile which has been secreted is reabsorbed, creating in effect an intrahepatic obstructive jaundice. It is obvious that mechanical obstruction of the ducts may lead to diffuse liver injury, particularly if associated with infection. It is probable also that some degree of liver injury must be present to produce jaundice in hemolytic anemias, since 95 per cent of a normal dog's liver may be excised without producing jaundice. This may be due to anoxemia resulting from the anemia (Rich).

Bilirubin which has been excreted by the liver and reabsorbed appears to differ in two important respects from that which has not passed through the liver cells, in that it gives a positive direct van den Bergh reaction and is readily excreted by the kidney, whereas the latter, which is believed to be combined in some way with plasma protein (or present in the form of a colloid, instead of a crystalloid), gives only an indirect reaction and is not excreted in the urine unless the concentration in the plasma becomes very high. A "biphasic" reaction is explained by the simultaneous presence of both types of bilirubin. If these views are valid, the otherwise puzzling behavior of the van den Bergh reaction can be more easily understood. The qualitative van den Bergh reaction, therefore, detects and differentiates between "free" (crystalloid) and "combined" (colloid) bilirubin and thus yields information of great diagnostic value. The quantitative van den Bergh measures the total bilirubin (of both types) present.

Van den Bergh Test: QUALITATIVE TEST. (1) In each of 3 small tubes put 0.25 ml. of clear serum or plasma (which must be free from hemoglobin). The serum must be examined within two hours or kept on ice. (2) To the first tube add 0.2 ml. water, and to the second tube add 0.2 ml. freshly mixed diazo reagent, shake, and wait 5 to 10 minutes. (3) If any color appears add 0.2 ml. of diazo reagent to the third tube and observe constantly, noting when the color first appears, and when it becomes maximal (use the first two tubes for comparison). The development of the maximal color in tube 2 can be hastened by adding a crystal of caffeine-sodium-salicylate. (4) If no color appears in five minutes add 0.5 ml. 95 per cent alcohol, shake, and note whether any color has appeared. Centrifuge if necessary.

The reaction can be made more sensitive by layering the reagent over the serum and observing the color at the plane of contact.

INTERPRETATION. An immediate direct reaction is indicated by the prompt appearance of a purplish-red color which becomes maximal within 30 seconds. A biphasic reaction is indicated by the appearance within 30 seconds (prompt biphasic) or 60 seconds (delayed

biphasic) of the red color, which gradually increases in intensity. A reaction which does not appear until more than a minute has elapsed has about the same significance as a positive indirect reaction.

If no color appears within one minute, but does appear (promptly) after adding alcohol, this constitutes a positive indirect reaction. Any serum giving a positive direct reaction will also give a positive indirect reaction.

Quantitative Test. (1) In a graduated centrifuge tube put 1 ml. clear serum. (2) Add 0.5 ml. diazo reagent and shake. (3) Add 2.5 ml. 95 per cent alcohol and shake. (4) Add 1 ml. saturated ammonium sulfate solution, mix, and let stand 15 minutes. (5) Centrifuge until clear. (6) Compare with standard solution in a colorimeter or comparator.

Calculation:

$$\frac{\text{Reading of standard}}{\text{Reading of unknown}} \times 4 \times 0.5 = \text{mg. bilirubin per 100 ml.}$$

Some prefer to substitute for 4 in this formula the observed volume of the clear alcoholic solution above the precipitate in the centrifuge tube.

If a dilution type of colorimeter is used, the formula becomes:

$$\frac{\text{Volume of unknown}}{\text{Volume of standard}} \times 4 \times 0.5 = \text{mg. per 100 ml.}$$

If the unknown requires dilution, use a mixture of alcohol 2 parts and water 1 part.

Normal serum contains usually from 0.1 to 0.3 (never over 0.5) mg. per 100 ml. Van den Bergh's "unit" corresponds to 0.5 mg. per 100 ml.

Reagents: ehrlich's diazo reagent: *Solution 1.* Sulfanilic acid, 1.0 Gm.; concentrated hydrochloric acid, 10 ml.; water, to 200 ml.

Solution 2. Sodium nitrite 0.5 Gm. in 100 ml. water. Renew once a week.

Immediately before use add 0.1 ml. of solution (2) to 10 ml. of solution (1).

standard solution. Dissolve 2.161 Gm. anhydrous cobalt sulfate (or if this is not obtainable, 3.92 Gm. of the recrystallized salt) in 100 ml. water to which 0.5 ml. concentrated sulfuric acid has been added. This keeps indefinitely in the dark. It corresponds in color to 0.5 mg. of bilirubin in 100 ml.

The color of the alcoholic solution may differ considerably in tint from that of the cobalt sulfate, and a better match may sometimes be obtained by substituting for the latter a solution prepared by diluting 0.7 ml. N/10 potassium permanganate to 50 ml. with distilled water. This must be freshly prepared.

Significance. In obstructive jaundice a positive direct reaction is obtained (occasionally with some "biphasic" intensification). A positive direct reaction may be given even by a trace of uncombined bilirubin, insufficient to raise the icterus index significantly. In hemolytic anemias an indirect reaction is usual. In diffuse disease of the liver any type of reaction may be obtained, depending upon the stage and severity of the process. Thus in catarrhal and arsphenamine jaundice the reaction is at first indirect; later it becomes biphasic; and if necrosis is extensive it may become direct. If recovery occurs, the same changes occur in reverse order, and this may be the first favorable prognostic sign. The reaction is, therefore, not of help in distinguishing jaundice due to mechanical obstruction of the ducts from other types. It is not a sensitive indicator of liver injury, since many cases occur without hyperbilirubinemia.

Icterus Index. This measures the intensity of the yellow color of the serum.

As this color depends largely upon bilirubin, the test is of some practical value in giving a simple approximate estimation of the bilirubin present. Hemoglobin, hematin, carotin and other lipochromes, if present, cause gross errors. The yellow pigments can be avoided by using a fasting blood specimen. A normal icterus index excludes hyperbilirubinemia. However, high values are much less dependable and should always be confirmed by the van den Bergh test. It is useful chiefly in following the course of a case from day to day.

Procedure. Procure clear serum or plasma without the slightest trace of hemoglobin (the chief source of error). Put 1 ml. serum in a comparator tube, and dilute with 0.85 per cent salt solution until the color matches that of a 1 : 10,000 solution of potassium bichromate (to which sulfuric acid has been added in the proportion of 4 drops to 1 liter, as a preservative). The volume of dilute serum in ml. gives the icterus index. Attempts to get more exact readings with a colorimeter are useless and imply a degree of precision which the test does not possess.

If the serum is tinged with hemoglobin or is cloudy, put 2 ml. serum in a graduated centrifuge tube, and add 4 ml. acetone. Mix, and centrifuge after five minutes. Note the volume of the supernatant solution, correct for dilution. Compare with standard solution, as above.

If carotin or other lipochromes are suspected, they may be extracted as follows: Put 2 ml. 95 per cent alcohol in a centrifuge tube, and add drop by drop 2 ml. serum, shaking constantly. Add 2 ml. petroleum ether, mix thoroughly, and centrifuge. The pigments are extracted in the supernatant ether layer, and the intensity of color may be compared with that of the potassium bichromate standard for icterus index determination.

Significance. Normal serum gives readings from 4 to 7 (maximum of 9). Readings from 10 to 16 may indicate "latent jaundice," while higher readings are obtained with evident jaundice (up to 100 or more). There is only an approximate correlation between these figures and those of the van den Bergh test. The icterus index figures may vary from 6 to 25 (usually about 10) times those of the van den Bergh test (expressed in mg. per 100 ml.). This discrepancy is due in part to the fact that the same amount of bilirubin in the uncombined (crystalloid) form imparts more color to a solution than it does in the combined (colloid) form.

Urobilinogenuria. An increased excretion of urobilinogen in the urine has been utilized as a test of liver function. Normally the greater part of the pigment which is absorbed from the intestine is taken up by the liver and re-excreted in the bile as bilirubin, only traces reaching the kidney. Marked increases are frequently seen in hemolytic anemias and in some cases of diffuse liver injury. However, experience has shown that this is not an early sign of liver injury and is often negative in cases with advanced injury. It has been found increased in about 20 to 25 per cent of miscellaneous cases of definite liver disease. An increase in the proportion of the total urobilin excreted which appears in the urine is more significant (see p. 855). In complete obstructive jaundice the traces normally present disappear (except with acute infections of the bile passages). (For methods see p. 835, Examination of the Urine.)

Bromsulfalein Dye Excretion Test (Phenoltetrabromphthalein Disodium Sulfonate). This dye has largely supplanted those previously used and is, on the whole, the most satis-

factory. Neither this nor any other excretion test is of any significance in cases with obstructive jaundice (when the serum yields either a direct or biphasic van den Bergh reaction). The dye may be purchased in 5 per cent solution in ampules ready for use (and also a comparator with standard color tubes) from Hynson, Westcott and Dunning, Baltimore).

Procedure. (1) Weigh the patient, and calculate the number of ml. required to furnish a dose of 5 mg. per kilogram (divide the weight in kilograms by 10, or in pounds by 22). (2) Inject this quantity, undiluted, into a vein, slowly (one minute), and carefully to avoid leakage. (3) At precise intervals of 5 minutes, 30 minutes, and 60 minutes withdraw 5 ml. of blood from the opposite arm vein, taking the usual precautions to prevent hemolysis. The 30-minute specimen is the significant one, and the others may be omitted in ordinary routine tests. (4) Put about 1 ml. of clear serum in each of two comparator tubes. To one add 1 or 2 drops of 10 per cent sodium hydroxide to secure maximum depth of color, and to the other 1 drop of 5 per cent hydrochloric acid. (5) Read at once in the comparator, backing the standard tube with the tube containing acidified serum, and the alkalinized serum tube with a tube of water.

Standard solutions may be prepared (in advance) by adding 5 mg. of the dye (0.1 ml. of the 5 per cent solution) to 50 ml. of water which has been alkalinized with 0.25 ml. of 10 per cent sodium hydroxide. From this solution, which corresponds to 100 per cent, make progressive dilutions with similarly alkalinized water (80, 70, 60, 50, 40, 30, 20, 15, 10, 5 per cent). Readings must be made promptly after the serum has been alkalinized, as clouding may occur after a few minutes and spoil the readings.

Interpretation. The normal reading five minutes after injection is from 20 to 50 per cent (average 35 per cent). After 30 minutes all the dye, except perhaps a trace, has disappeared. The presence of 10 per cent or more of the dye 30 minutes after the injection indicates definite impairment of function. In cases of grave liver injury considerable amounts may persist for several hours. Some advise an interval of 45 minutes instead of 30 minutes.

The dose of 2 mg. per kilogram originally advised is much less efficient as a test. If this dose is used, the blood should be obtained 20 minutes after the injection.

Significance. The test is not a sensitive indicator of milder degrees of liver injury. Positive evidence of impairment has been obtained in about 60 per cent of cases of proved liver disease, particularly in cirrhosis (but much less frequently if a dose of 2 mg. per kilogram is used). This percentage is as large as, or larger than, that given by any other test except the bilirubin excretion test. There is no close correlation between the amount of dye retained and the gravity of the disease, and a normal result does not exclude liver disease.

Bilirubin Excretion Test (von Bergman). This offers the obvious theoretical advantage that it tests the reserve capacity of the liver to perform one of its natural functions under an overload. It is useless in any patient with hyperbilirubinemia. It is a highly sensitive test, revealing impairment of function in a larger proportion of patients with liver disease than any of the other usual tests. It is somewhat complicated to carry out, however, and thus far has not come into general use. For technic see Soffer and Paulson (1934).

Other Tests of Liver Function

Hippuric Acid Excretion Test (Quick). (1) After a light breakfast, give the patient a glass of water, and have him empty the bladder. (2) Inject intravenously 1.88 Gm. sodium benzoate dissolved in 20 ml. sterile distilled water (prepared with the usual precautions), taking five minutes or more for the injection. This corresponds to 1.5 Gm.

of benzoic acid. (3) Just one hour after completing the injection collect the entire amount of urine, by catheter if necessary. (4) Acidify with acetic acid if the analysis is not completed at once. (5) Measure the volume, and if over 100 ml., concentrate the acidified urine over a water bath to a volume of 50 to 100 ml. (6) If less than 100 ml., heat the acidified urine over a boiling water bath, in either case adding 1 teaspoon of powdered charcoal if it is high colored or contains bile. (7) Filter while hot through coarse and then through fine filter paper. (8) Add 5 Gm. ammonium sulfate for each 10 ml. filtered urine, heating if necessary to dissolve. (9) Cool and acidify with strong hydrochloric acid until it gives a strong reaction with Congo red paper. Stir with a glass rod, scratching the sides of the flask to start crystallization, and let stand until crystallization is complete, overnight if necessary. (10) Filter by gentle suction through a fine filter paper. (11) Wash with 3 portions of 5 ml. ice water. (12) Dry in incubator at 37° C. overnight. (13) Weigh, subtract the weight of the filter paper, and add 0.1 Gm. for each 100 ml. solution, including the washings, to compensate for dissolved hippuric acid.

The hippuric acid is formed by the conjugation of benzoic acid with glycine which is synthesized in the liver. (The conjugation occurs in both the liver and kidney.) The maximum amount of hippuric acid which can be formed from the sodium benzoate given is 2.2 Gm. Normally 1 Gm. or more is excreted during the first hour. A smaller excretion indicates impairment of liver function. The test is relatively sensitive, but is unreliable if renal function is impaired or urinary excretion otherwise disturbed.

Galactose Tolerance Test. Theoretically this sugar is an ideal test substance, since it is not utilized by muscle, and since normally all is taken up by the liver and converted into glycogen without need of insulin. Any that passes through the liver is quantitatively excreted by the kidneys, since the renal threshold for galactose is believed to be zero. It is not necessary to follow the blood sugar. The test may be used even in diabetics, and is especially useful in jaundiced patients.

Procedure. Administer to the patient, fasting, 40 Gm. of chemically pure galactose in 250 ml. (or more) of water. Collect all the urine in separate hourly specimens for five hours, giving water as desired, but no food. Test each specimen for sugar (Benedict's method). Combine any specimens which contain sugar, and estimate the sugar quantitatively, as glucose. If glucose is present it may be removed entirely, without appreciable loss of galactose, by rapid fermentation. Make up a 10 per cent suspension of thoroughly washed yeast. To 7.7 parts of this add 1 part of urine and incubate for 45 minutes. Determine the quantity of sugar remaining, all galactose (Shay et al., 1931).

Significance. A decreased tolerance is indicated by a total excretion of more than 3 Gm. of sugar. The chief value of the test is in the differentiation of obstructive from nonobstructive jaundice. It is generally agreed that impaired tolerance is rare in the former group, but common in the latter (catarrhal jaundice, etc.). In other types of liver disease the test is of little value, as it is rarely positive, and usually only in the advanced stages.

Cephalin-cholesterol Flocculation Test (Hanger). (1) In a centrifuge tube put 0.2 ml. serum (which is fresh or has been preserved in the icebox). (2) Add 4 ml. 0.85 per cent salt solution. (3) In a similar tube put 4 ml. salt solution only. (4) To both tubes add 1 ml. cephalin-cholesterol emulsion. (5) Mix, stopper with cotton, and let stand upright at room temperature for 48 hours. Read at 24 and 48 hours. Record as negative if there is no change in the suspension. The degree of precipitation, if any, is recorded as from + to $+^4$, the latter being complete precipitation with clear supernatant fluid.

Individuals without hepatic disease give a negative reaction. Patients with obstructive

jaundice usually give at most a weak positive (+) reaction, which presumably indicates some secondary liver injury. Patients with significant diffuse liver injury (cirrhosis, hepatitis, arsphenamine jaundice, etc.) give a prompt strong positive reaction. Negative reactions are obtained in hemolytic jaundice, in circumscribed liver lesions including most cases of carcinoma, and in some cases of jaundice following arsenicals, etc. The test is relatively sensitive, and may give positive reactions in other diseases such as severe infections, leukemia, uremia, in which it is difficult to demonstrate liver injury by other methods.

CEPHALIN-CHOLESTEROL: STOCK SOLUTION. Dissolve 100 mg. sheep brain cephalin and 300 mg. cholesterol in 8 ml. ether. Stopper tightly and shake until completely dissolved. (A suitable preparation can be purchased from Difco Laboratories.)

WORKING EMULSION. In a test tube graduated at 15 ml. put 17.5 ml. distilled water, warm to 65° to 70° C. in a water bath. Add 1 ml. of the stock solution slowly, stirring constantly. Bring to a boil slowly and let simmer until the volume is reduced to 15 ml. Cool. (This gives best results if freshly prepared but can be stored for a short time if merthiolate is added to make a concentration of 1 : 10,000.)

Cholesterol Esters. The formation of cholesterol esters from cholesterol and fatty acids is believed to take place in the liver, and a reduction in the percentage of blood cholesterol which is present in ester form is regarded as an indication of liver injury. (See p. 791.)

Plasma Proteins. A reduction in total protein and particularly in plasma albumin is frequently observed in severe liver disease. This also occurs, however, in other diseases in which the liver is not notably involved, such as "nephrosis," malnutrition, and certain infections.

Prothrombin Formation. In patients with a prolonged prothrombin time, particularly if jaundiced, failure to secure improvement by administration of vitamin K is an indication of severe liver injury. (See p. 467.)

Other Chemical Changes. In the most advanced (premortal) stages of liver necrosis the following changes may be observed:

1. Fall in blood urea nitrogen, with a rise in amino-acid nitrogen and total nonprotein nitrogen.
2. Fall in urinary urea, with increase in ammonia and amino-acid excretion, and occasionally the appearance of leucin and tyrosin crystals in the sediment.
3. Reduction of fibrinogen in the blood and a tendency to bleed.
4. Marked hypoglycemia.

CHAPTER 43

The Endocrine Glands

Procedures are available for titrating in blood or urine the quantity of a number of hormones or their derivatives, and thus obtaining a measure of the secretory activity of the glands secreting them. These are mostly complicated procedures requiring the facilities of a well-equipped laboratory. Of the hormone tests only the more usual tests for pregnancy will be discussed here.

Basal Metabolism

By basal metabolism is meant the total energy produced by the combustion of food substances in the body under conditions which reduce the energy expenditure to a minimum; i.e., when the individual is fasting and is physically and mentally at rest and relaxed. The metabolism is raised above the basal level by the chemical processes involved in digestion and absorption (10 to 15 per cent) and by mental or physical activity.

The metabolism may be determined directly by measuring the heat liberated in a calorimeter, or it may be calculated if the oxygen consumed and the carbon dioxide liberated (and the nitrogen excreted in the urine) over a fixed period are measured accurately. The amount of heat liberated for each liter of oxygen consumed ("caloric value" of O) (or carbon dioxide produced) is not a constant but depends upon the relative amounts of carbohydrate, fat, and protein in the food mixture oxidized. If carbohydrate only is burned, for each atom of carbon burned, one molecule of oxygen is consumed and one molecule of carbon dioxide is produced. The respiratory quotient (R.Q.) or the ratio by volume of the CO_2/O involved is, therefore, 1.0. The caloric value of one liter of oxygen (and also of carbon dioxide) under these circumstances is found by calculation and observation to be 5.05 calories. If fat only is burned, aside from the oxygen required to oxidize the carbon to carbon dioxide, additional oxygen is needed to oxidize the hydrogen, which is eliminated as water. The ratio of CO_2/O is therefore less than 1.0 (for average fat 0.707) and the caloric value of one liter of oxygen is less than that of one liter of carbon dioxide. (O, 4.69 cal.; CO_2, 6.63 cal.) Under all ordinary conditions fat and carbohydrate are both burned, in varying proportions, as well as some protein (which has a R.Q. of about 0.80). To estimate the energy output precisely in this way is a tedious procedure.

It has been found, however, that *in the fasting state* if the subject has been taking an average diet, the R.Q. is relatively constant, on the average 0.82, and that for routine diagnosis (except in diabetics of appreciable severity) no error of practical significance is introduced if this average figure is assumed. It is, therefore, possible to calculate the basal metabolism with sufficient accuracy by determining either the oxygen consumption or the carbon dioxide output. Both procedures have been employed. Calculations based on oxygen consumption are more reliable than those based on carbon dioxide excretion alone. The variation in the caloric value of carbon dioxide is much greater than that of oxygen if the actual R.Q. varies from the average figure. There may

also be a "pumping out" of carbon dioxide from the plasma bicarbonate if the pulmonary ventilation rate is increased (or a storage of carbon dioxide if the rate is diminished) during the brief period of the test. The caloric value of a liter of oxygen with a R.Q. of 0.82 is 4.825 calories.

Methods. If both oxygen consumption and carbon dioxide elimination are to be measured and the R.Q. precisely calculated, the Tissot "open method" should be used. This is practicable only in well-equipped laboratories with specially trained technicians, and the procedure will not be described.

For determinations of oxygen consumption alone, several satisfactory simple "closed circuit" outfits are available. Among those most used are the Sanborn, the Roth, and the Collins modifications of the original "portable" apparatus of Benedict; the Krogh; and McKesson's Metabolor. With all of these, the nose of the patient is closed with a clip, and he breathes through a suitable mouth piece and breathing tubes into an air chamber sealed with a water jacket and containing an excess of oxygen. The expired air is carried through a jar of soda lime inside the chamber to remove carbon dioxide. The circulation of air may depend upon the patient's own efforts, controlled by flutter valves, or upon a motor blower. By means of a kymograph variations in the volume of air in the apparatus are recorded on special graph paper, from which the diminution in volume (oxygen consumed) can be read directly. This is corrected for temperature, changes in temperature if any, barometric pressure and moisture (the air is 80 per cent saturated), and converted into energy equivalents (calories per hour) by means of tables which are furnished with the apparatus, together with detailed instructions for its operation.

Accurate results depend upon scrupulous attention to details, of which the following are especially important. The test must be carried out in the morning with the patient under "basal" conditions. He must fast for 14 hours before the test. In the morning, before the test, he should not smoke, drink coffee or tea, or make unnecessary physical exertion. A glass of water may be allowed, as this is less disturbing than discomfort from thirst. As a rule the patient may come to the laboratory for the test if he rests at least half an hour before the test. The room must not be cold or excessively hot. The procedure should be explained in detail. Apprehension, restlessness, or discomfort of

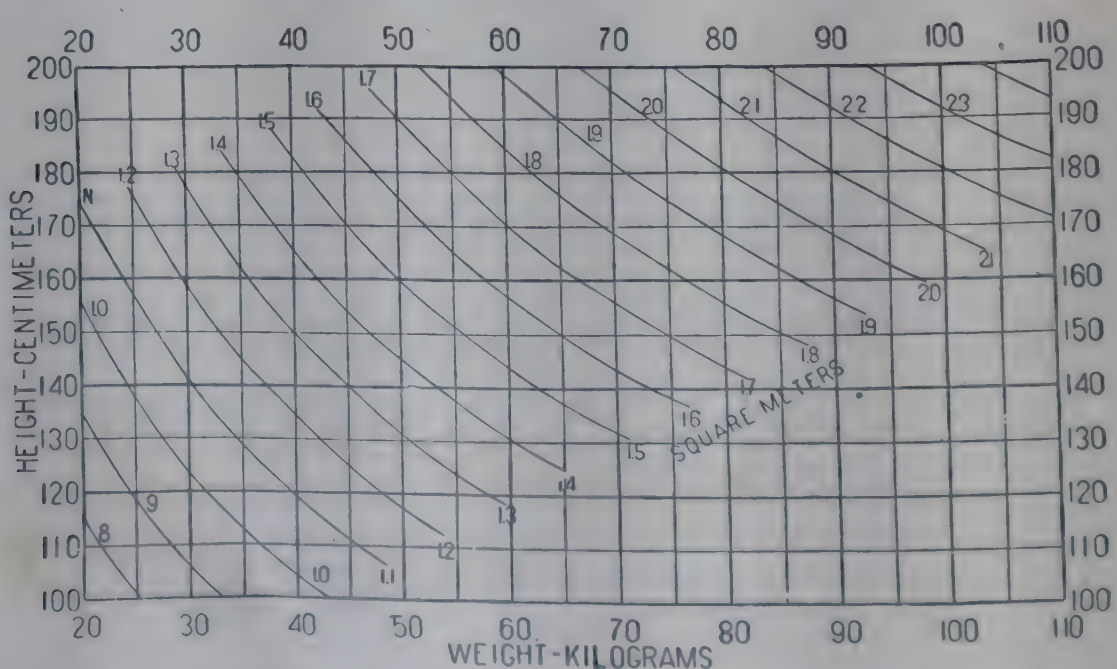


Chart for the estimation of the surface area of the body from the height and weight. Plotted from the DuBois formula. (Courtesy, E. F. DuBois, Basal Metabolism in Health and Disease, Philadelphia, Lea and Febiger.)

any sort increases oxygen consumption and leads to gross errors. Complete coöperation of the patient is indispensable. Practically all errors tend to make the result too high. Two periods of 6 to 10 minutes should be tried. If the results are discordant, the lower figure is usually more nearly correct. A third period may be tried, but as a rule it is better to dismiss the patient and repeat the test the following day. This should always be done if the result is pathologically high, if it is higher than was expected, or if the test appears to be unsatisfactory in any way.

Errors inherent in the use of the apparatus are unusual if reasonable care is taken to avoid them. The most common are: leakage of air, especially around the mouth piece or nose clip, rarely through a perforated ear drum; obstruction to free circulation of air (due to water in the breathing tubes, sticking valves, or caked soda lime); and incomplete absorption of carbon dioxide because of too protracted use of the soda lime. The latter may give too low a result, but usually causes dyspnea and irregular breathing. Test occasionally by forcing air out through the oxygen intake tube into a clear solution of barium hydroxide. If clouding occurs, renew the soda lime.

Table 80

NORMAL STANDARDS OF AUB AND DUBOIS
In Calories per Square Meter of Body Surface per Hour

Age	*Males*	*Females*
14–16	46.0	43.0
16–18	43.0	40.0
18–20	41.0	38.0
20–30	39.5	37.0
30–40	39.5	36.5
40–50	38.5	36.0
50–60	37.5	35.0
60–70	36.5	34.0
70–80	35.5	33.0

Table 81

BENEDICT-TALBOT STANDARDS FOR CHILDREN
In Calories per Hour from Body Weight

Weight, Kg.	*Boys, cal.*	*Girls, cal.*	*Weight, Kg.*	*Boys, cal.*	*Girls, cal.*
3	6.3	6.3	21	36.9	34.6
4	8.8	9.2	22	37.9	35.6
5	11.3	11.9	23	39.2	36.7
6	13.8	14.6	24	40.2	37.5
7	16.3	16.9	25	41.3	38.8
8	18.5	19.2	26	42.5	39.6
9	20.6	20.8	27	43.5	40.6
10	22.7	22.5	28	44.6	41.7
11	24.6	24.2	29	45.4	42.5
12	26.0	25.4	30	46.5	43.5
13	27.5	26.7	31	47.5	44.6
14	29.0	27.7	32	48.3	45.4
15	30.2	28.8	33	49.2	46.4*
16	31.5	29.6	34	50.0	47.3*
17	32.5	30.6	35	50.8	48.3*
18	33.5	31.7	36	51.7	49.2*
19	34.6	32.5	37	52.3	50.1*
20	35.8	33.5	38	53.1	50.7

*These figures obtained by interpolation.

Significance. The basal metabolism in normal individuals varies with their age, sex, and size. It varies more nearly with the surface area than with either height or weight. The area may be calculated, from the height and weight, by the DuBois formula:

$$\text{Area} = \text{Height (cm.)}^{0.725} \times \text{Weight (kilograms)}^{0.425} \times 71.84$$

or read by interpolation from the chart.

The basal metabolism is usually expressed as the number of calories produced per square meter of body surface per hour. It is greatest in early childhood and gradually diminishes with age. It is from 7 to 10 per cent lower in women than in men. There is as yet no universally accepted *normal standard*. The three standards most extensively used are those of *Aub and DuBois*, based on surface area; of *Harris-Benedict*, based on height and weight; and of *Dreyer*, based on weight. We give the table of Aub and DuBois because this is in most general use, and is more dependable for subjects of unusual build. Their figures, which were based on tests in a calorimeter chamber lasting an hour or more, are about 6 per cent higher than those obtained by most workers by measuring oxygen consumption over 6- to 10-minute periods during which more complete relaxation may be maintained. For children the standards of Benedict and Talbot may be used. The basal metabolic rate is usually expressed as the percentage by which the observed figure deviates from the normal average. The standard with which it is compared should always be recorded. Figures from —10 per cent to +10 per cent (and usually to +15 per cent) are within the limits of the normal variation.

Recent studies (Shock, 1942; and others) indicate that the normal standards of Aub and DuBois for adolescents are also too high, particularly for females. Shock gives the following mean values:

Age 13: Males, 44.1 cal.; females, 39.9 cal.
Age 15: Males, 42.8 cal.; females, 35.7 cal.
Age 17: Males, 40.9 cal.; females, 33.4 cal.

Alterations in Disease. The B.M.R. is controlled primarily by the thyroid through its secretion of thyroxin. In severe cretinism and in myxedema and after total thyroidectomy the B.M.R. falls to a fixed level about 40 per cent below normal. It can be elevated with relative precision by intravenous injections of thyroxin, rising after a latent period of 12 hours 3 per cent for each 1.0 mg. administered. In cases of thyroid intoxication, whether due to exophthalmic goiter or toxic adenoma, the B.M.R. is elevated (+ 25 per cent to + 100 per cent), the degree of increase being proportional to the acuteness and severity of the disease. Repeated estimations of the B.M.R. afford the best single means of determining the results of treatment and the optimum time for operation.

Fever from any cause results in an elevation of the basal metabolic rate of about 7.2 per cent for each degree F. (13 per cent for 1° C.). Elevation of the B.M.R. is also seen in leukemia in the active stages, in many cases of polycythemia, in some cases of pernicious anemia, in some cases of pituitary disease with hyperplasia of the anterior lobe, and after the administration of certain drugs, particularly epinephrin, ephedrin, dinitrophenol and (in less degree) caffeine.

Mild degrees of *hypothyroidism* characterized by asthenia, easy fatigue, sensitiveness to cold, bradycardia, and constipation, with a B.M.R. of —15 per cent to 30 per cent, are common. They are usually benefited by the administration of thyroid in amount sufficient to raise the rate moderately toward the normal. A moderate reduction is common in malnutrition and cachexia from any cause. A reduction is seen also in a few cases of obesity, in hypopituitary conditions (Frohlich's syndrome, Simmond's disease),

in some cases of Addison's disease, in "nephrosis," and in some psychoses accompanied by markedly apathetic depressed states.

A few apparently healthy individuals are met with who show a B.M.R. substantially below the normal figure. If their rate is raised to the usual normal by treatment or by disease, they may show signs of thyroid intoxication. They are peculiar in that their metabolism appears to be set at an unusually low level.

Reed's Formula. In individuals with no abnormality of the cardiovascular system the resting pulse rate is usually proportional to the B.M.R. Reed has suggested the following formula for calculating the B.M.R. from the pulse rate and pulse pressure.

B.M.R. = 0.685 (Pulse rate + 0.9 Pulse pressure) — 71.5

Example: Pulse rate 100. Pulse pressure 70 (150 — 80).

$$\text{B.M.R.} = 0.685(100 + 0.9 \times 70) - 71.5$$
$$= +39.8 \text{ per cent}$$

Reed reported that the error was less than 10 per cent in 60 per cent of the cases and less than 20 per cent in 90 per cent of the cases.

Pregnancy Test

Aschheim-Zondek Test. This is not a direct test for pregnancy, but for the anterior pituitary-like gonadotropic hormone (prolan) which is present in the urine in large amounts during pregnancy. It is so arranged quantitatively that the (usually) small amounts present in other conditions are not revealed.

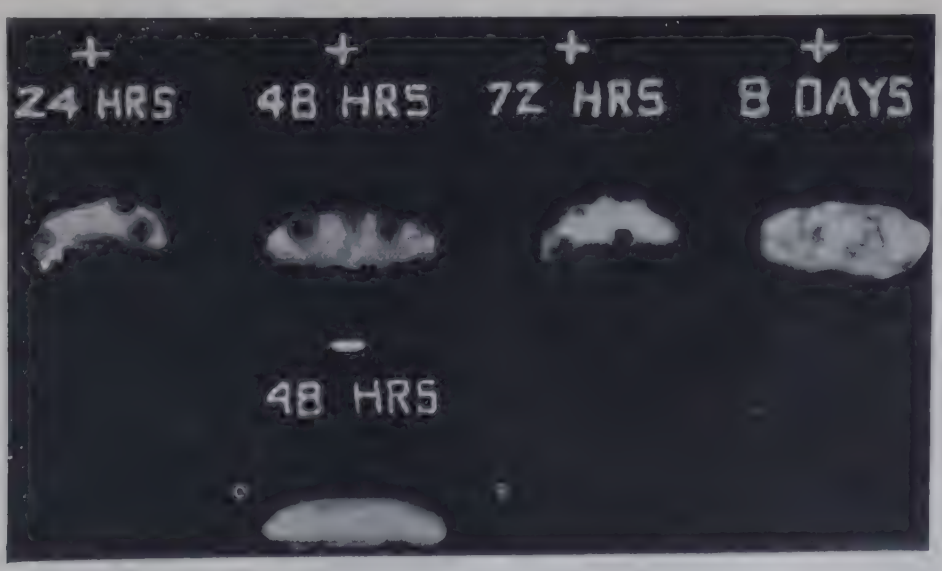
Procedure. Five infantile mice from three to four weeks old and weighing 6 to 8 Gm. are each given subcutaneous injections of 0.5 ml. of urine twice daily for three consecutive days. If the urine is toxic for the mice, this difficulty can sometimes be remedied by extraction with ether. About 96 hours after the first injection the mice are killed and the ovaries are examined with a hand lens for:

Reaction 1: Enlarged follicles.

Reaction 2: Hemorrhages into the follicles (Blutpunkte).

Reaction 3: Corpora lutea.

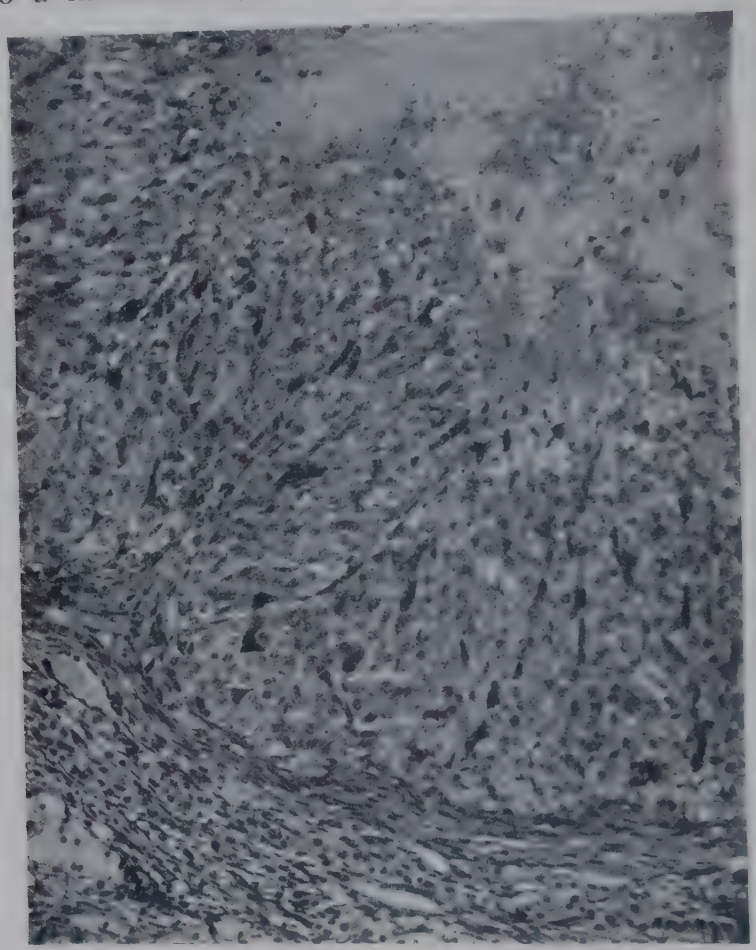
The occurrence of Reaction 1 only is suggestive, and the test should be repeated. Reaction 2 or 3 is definitely positive. Occasionally positive reactions may be evident after 60 hours. Aschheim and Zondek reported 99.5 per cent negative reactions in 1075 non-pregnant individuals, and 98 per cent positive reactions in 925 cases of pregnancy. Later work by many different investigators fully confirms their results and indicates that the error does not exceed 2 per cent.



Gross photograph. Rabbit ovaries. Natural size. Gross appearance of positive test of 1, 2, 3, and 8 days' duration and of negative test. (After A. M. Young, from *J. Lab. Clin. Med.*)

Friedman Test. Friedman (1929) recommended the use of a single mature female rabbit in place of the mice. The animal is isolated in an individual cage for three weeks, and then given 24 ml. of urine intravenously in divided doses. About 48 hours after the first injection the animal is killed and the ovaries examined. The presence of hemorrhagic follicles constitutes a positive reaction. This method with minor modifications has been used extensively in the United States with an error not exceeding 5 per cent.

We recommend the following procedure described by Young (1934), in order to reduce the errors to a minimum. (1) Select healthy female rabbits weighing 2 to 4

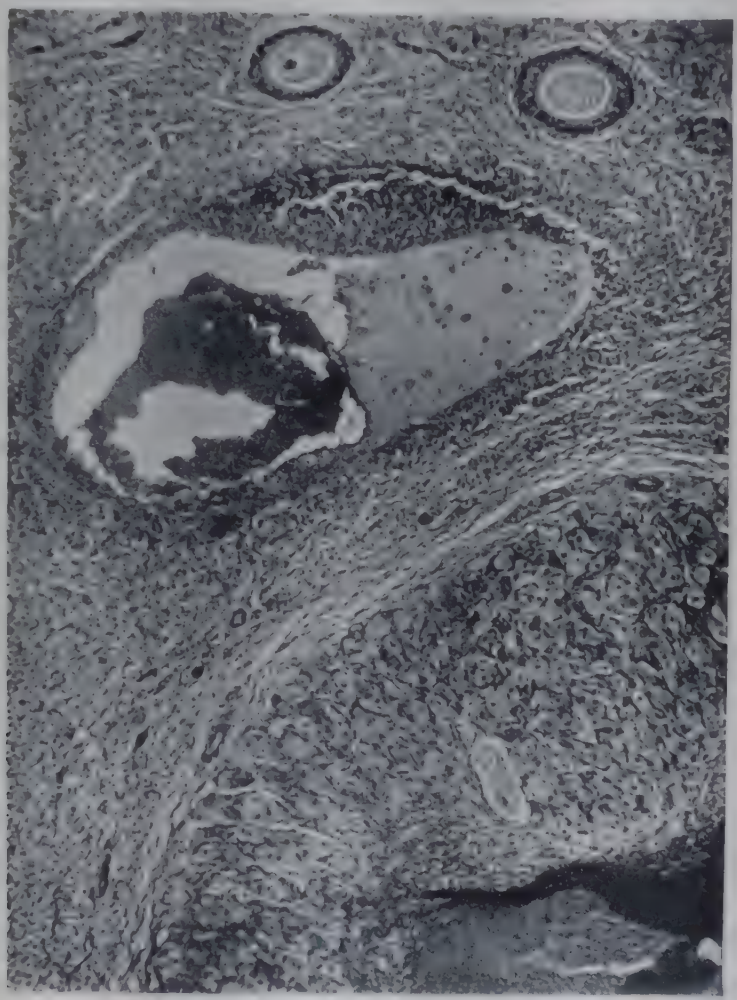


Rabbit ovary. Corpus luteum of 48 hours' duration showing proliferation of capillaries. (Photomicrograph, high power.) (After A. M. Young, from *J. Lab. Clin. Med.*)

kilograms. (2) Isolate in individual cages. (3) Under morphine anesthesia (½ gr. intravenously) open the abdomen and inspect the ovaries, selecting animals with well-developed follicles but without corpora haemorrhagica or corpora lutea. (4) Secure a specimen of urine voided in the morning with a specific gravity of at least 1.015. If necessary restrict fluids the previous day to secure the needed concentration. Filter clear, warm to body temperature, and slowly inject 15 ml. intravenously. Repeat the injection after four hours. (5) After 48 hours again inspect the ovaries. If they appear positive on gross inspection, remove one ovary and examine microscopically a frozen section. The presence of lutein tissue and some hemorrhage in one or more follicles constitutes a positive reaction. (6) If the ovaries appear normal, a tentative negative report may be made. Close the abdomen and inject as a control 15 ml. of known positive urine (or a suitable concentrate). (7) After 48 hours again inspect the ovaries. If corpora haemorrhagica are present, indicating that the animal was capable of a normal response, the reaction [in (6)] may be regarded as definitely negative.

If it is not possible to prepare sections, the ovaries should be inspected again on the fifth or sixth day. If positive, corpora lutea will then be unmistakable in gross. If the urine is toxic for the rabbits, the hormone may be concentrated by adding to 60 ml. of urine, 5 volumes of 95 per cent alcohol. Centrifuge in large tubes, decant, and dissolve the precipitate in a few ml. distilled water. Extract for 15 minutes with 3 volumes of ether; aerate to remove the ether, and inject the aqueous solution (volume of about 10 ml.) slowly into the vein.

If the preliminary inspection of the ovaries is carried out, the initial period of isolation of the rabbits may be shortened or omitted, although Young found 20 per cent of her



Rabbit ovary. Positive test showing follicles of 24 hours' and of 8 days' duration. (Corpus haemorrhagicum and corpus luteum.) (Photomicrograph, low power.) (After A. M. Young, from *J. Lab. Clin. Med.*)

stock animals unsuitable. Animals usually may be used a second time if they are isolated at least a month after the first test.

By the use of these precautions errors due to individual variations in the test animals are practically eliminated. In her series Young reported one false negative in 96 cases of pregnancy, and 4 false positives in 135 nonpregnant individuals, three of whom had teratomatous tumors of the ovary or testis. The test, therefore, compares favorably in accuracy with the original Aschheim-Zondek method.

The reaction usually becomes positive within a few days after the first missed menstrual period. Positive reactions have been reported 10 to 16 days after conception before a period has been missed. The reaction becomes negative within a week after delivery unless membranes are retained. It is positive in ectopic

pregnancy as long as intact chorionic villi are present. Disappearance of a positive reaction usually means death of the fetus.

Positive reactions are regularly found in cases of hydatidiform mole, chorionepithelioma, and teratomas containing chorionic epithelium in both men and women. The reaction becomes negative quickly after removal of the tumor. The persistence or reappearance of a positive reaction indicates incomplete removal or recurrence.

Frog Test. Bellerby (1934) reported that the South African clawed frog (*Xenopus laevis*) may be used as a test animal to detect the ovary-stimulating hormones present in the urine during pregnancy. The females carry eggs throughout the year but extrude them only after mating or after injections of material containing these hormones.

The females must be isolated from the males. Five frogs may be kept in a 10-gallon aquarium containing water to a depth of 3 inches. They should be in a well-lighted room, at a temperature between 20° and 25° C., and the water must be kept clean. They may be fed strips of raw heart or liver twice a week. They should not be fed for 24 hours before use. Frogs may be used repeatedly if given a rest of a week following a negative reaction and preferably a month following a positive reaction. It is preferable to use frogs which have reacted positively, or to repeat the test with such an animal if a negative reaction is obtained with a new frog.

Test. Obtain a fresh 4-ounce specimen of urine, preferably a first morning voiding. Untreated fresh urine can be used, but better results are obtained by concentration (Weisman, et al., 1942). To 80 ml. urine, acidified to litmus with acetic acid if necessary, add 160 ml. acetone and let stand 15 minutes. Centrifuge and discard the supernatant fluid. (The acetone can be recovered by distillation.) Wash the precipitate twice with 20 ml. ether and dry it thoroughly. Suspend the precipitate in 2 ml. water, and centrifuge. Just before use decant the clear fluid, and bring the pH to 5.5 with 10 per cent sulfosalicylic acid, using nitrazine paper as indicator.

Inject 1 ml. of the clear fluid into the dorsal lymph sac of one (or each of two) frogs. Using a long slender hypodermic needle, insert the tip under the skin of the dorsal surface of the left thigh, and keeping the point just beneath the skin, push it forward and inward till it reaches the midline above the cloacal fold. It may then be forced forward into the lymph sac, taking care not to puncture the lung. Inject the material and withdraw the needle.

Put the frogs into individual 2-gallon containers with 3 inches of water in the bottom and provided with a one-half inch mesh wire screen raised 1 inch above the bottom of the container to prevent the frogs from eating the ova. Inspect at intervals from 4 to 18 hours after the injection. In the case of a positive reaction, the frog usually deposits large numbers of ova easily seen macroscopically within nine hours and often within six hours. If none are deposited within 18 hours, the test is negative. Before being used again, the wire screen should be heated in a free flame to burn off any ova adhering to it.

Available reports indicate that if the test is properly performed, the error does not exceed 2 per cent. It appears, therefore, to be fully equal in reliability to the preceding methods in which mice or rabbits are employed. It is perhaps a little more sensitive, and is far superior in simplicity, economy and the speed with which the result can be obtained. All of these tests may give positive reactions in certain relatively uncommon conditions other than pregnancy, in which the anterior pituitary hormones are increased.

CHAPTER 44

Vitamins as Specific Food Factors: Their Nutritional Significance and Deficiency Effects

Considerable attention has been given to man's requirements for specific dietary essentials. The recommended daily allowances of the National Research Council provide considerable margin of safety, develop nutritional reserves, and obviate any possibility of malnutrition.

Lord Horder[1] has pointed out that considerable overemphasis has been placed on "balanced" diets and the need for specified daily vitamin intakes. Platt,[2] Dann,[3] and Keeton and Mitchell[4] have corroborated this, and feel that the need for specific amounts of the dietary essentials is open to question.

Specific avitaminoses are clinically rare and generally result from long periods of deficit. Certain methods of diagnosing avitaminoses have recently been questioned and evaluation of dietary effects requires much care.[5] Recent reports indicate no close correlation between dark adaptation of adults and vitamin A,[6] and it is difficult to improve the dark adaptation of subjects poor in this respect with even huge doses of vitamin A. Gingivitis, often attributed to scurvy, is more often not related to this disease[7]; beriberi will not appear for months in subjects with decreased thiamin intakes if they are initially saturated.[8] The vascularity of the cornea occurring in rats deprived of riboflavin does not occur in monkeys; they develop a patchy dermatitis instead.[9] In man sufficient data are accumulating to show that corneal vascular lesions are rarely the result of ariboflavinosis.[10] Cheilosis may sometimes be due to riboflavin deficiency, but there are many other causes for it. Research is still needed to determine classical clinical symptoms for a clear diagnosis of some of the avitaminoses.

It has been shown, furthermore, that the requirement for any one nutrient is dependent on the presence of others[11] with respect to "metabolic mixture." The interrelationships of nutrients and individual needs which depend upon environment and function complicate the interpretation of nutritional surveys.

The question of bulk in the diet is of importance from the point of view of weight control as well as in its effect on intestinal biosis and motility. The maintenance of beneficial intestinal bacteria capable of synthesizing folic and pantothenic acids is of importance especially as these bacteria further synthesize a portion of the niacin, riboflavin, and thiamin requirements.

Vitamins

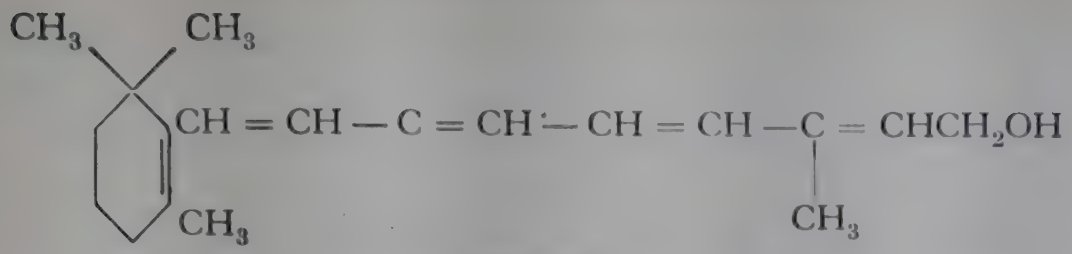
The vitamins or accessory food factors in a nutrient program can, in view of the rapid advance in our knowledge of chemistry, be classed as pharmacologic

substances. From unknown dietary constituents vitamins have evolved into definite chemical entities with marked physiologic and therapeutic activity. They function as parts of enzyme systems (B_1, B_2, niacin and ascorbic acid); in the regulation of metabolism of certain minerals (vitamin D); or in the constitution of certain protein complexes (vitamin A in rhodopsin). Considered as such they can conveniently be classified as drugs. This is especially significant when we consider the fact that body economy is supported to a great degree, all things being equal, by adequate vitamin intake. Such vitamin consumption can be regarded as a *maintenance dose*. On the other hand, if a marked deficiency exists, or for other reasons large doses are to be administered, these can be regarded as *curative* and *therapeutic* doses.

The indiscriminate use of vitamins is to be deplored, as there is no clear-cut evidence which indicates that the vitamins are of therapeutic value in diseases uncomplicated by vitamin deficiencies. The current exaggerated interest in vitamins has produced remarkably little acceptable evidence for their clinical value except in conditions of definite avitaminosis. There is, on the other hand, a dangerous tendency to apply vitamins to the treatment of undiagnosed complaints having their counterpart in frank deficiency disease. Many of the associated symptoms in patients—weakness, muscle pain, anorexia, constipation or diarrhea, fatigue—have their analogues in certain true vitamin deficiencies, but similar therapy on this ground alone is unwarranted. Regardless of nutritional requirements in normal metabolism, in the pharmacologic use of vitamins a therapeutic result is desired. In short, therapy is instituted with a drug, in this case a vitamin, for some specific need. On this basis it is obvious that diagnosis is essential and that if deficiency disease is present vitamin therapy is indicated. It is well to bear in mind that in the majority of instances the vitamin deficiency syndrome is the result of a lack of several vitamins. This makes for the bizarre clinical syndromes too often seen in undernourished peoples. Thus the salient deficiency is noted and treated intensively, other vitamins and nutrients are given also as a supportive measure.

Vitamin A: Antixerophthalmia, Antixerosis, Antikeratomalacia

Xerophthalmia and keratomalacia are probably late developments of vitamin A deficiency. Symptoms occur with greater ease in children than in adults. In fact, vitamin A deficiency of adults has never been adequately studied and many lesions described are presumably old ones and are irreversible. The body reserves of vitamin A are extraordinary, and extreme dietary deficiency is usually inconsequential in provoking symptoms specific for deprivation of this vitamin. However, there are certain pathologic conditions, superimposed on a background of low vitamin A intake, where absorption is poor and where liver damage may exist. Thus certain symptoms of avitaminosis A can occur in liver disease and jaundice, and it may even be related to a faulty metabolism in the conversion of certain carotenes to vitamin A. Mineral oil furthermore interferes with the absorption of carotene and diarrheas of all types, especially steatorrhea, promote the loss of the vitamin in the feces.



Vitamin A

Where there are signs of vitamin A deficiency (hyperkeratosis and other epithelial changes) the question of dosage is a real one. Doses of 50,000 to 300,000 units are of no greater value than doses of 15,000 units. It is well to bear in mind that the processes of tissue repair are slow and are not enhanced by increasing the dosage. A daily supplement of 15,000 units has been found to be as effective as much larger doses in children with vitamin A deficiency.

	International Units
Average daily requirements in children (empirical)	3000 to 6000
Average daily requirements in adults is unknown (empirical)	2000 to 4000
During pregnancy and lactation (empirical)	4000 to 7000

Claims for vitamin A in the treatment of color blindness are denied, and the dark-adaptation test for vitamin A deficiency becomes increasingly untrustworthy. The presence of Bitot's spots may be the result of an early vitamin A deficiency to which superimposed effects (sunshine, wind, etc.) are contributory.

Sources:

	International Units
Carrots (raw) per ounce	1000
Butter " "	600
Cheese " "	1000
Eggs " "	1000
Liver " "	3000
Milk " "	29–183
Spinach " "	3000

Pharmaceutical preparations:

	U.S.P. Units per Gm.
Halibut liver oil	44,800
Cod liver oil (refined)	1,800
Cod liver oil (crude)	850
Cod liver oil (concentrated)	14,000 to 60,000
Percomorph liver oil	60,000
Shark liver oil	16,500

Pathology in Avitaminosis A. The primary lesion in vitamin A deficiency consists of a metaplasia of the ectodermal structures with a transformation of the normal epithelium into stratified keratinized epithelium. This process involves the eye, the lacrimal glands, the salivary glands, the mouth and throat, parts of the respiratory tract (larynx, trachea, bronchi), the gastrointestinal tract, the genitourinary tract, and even the skin, which may become dry, scaly, shriveled, and pigmented.

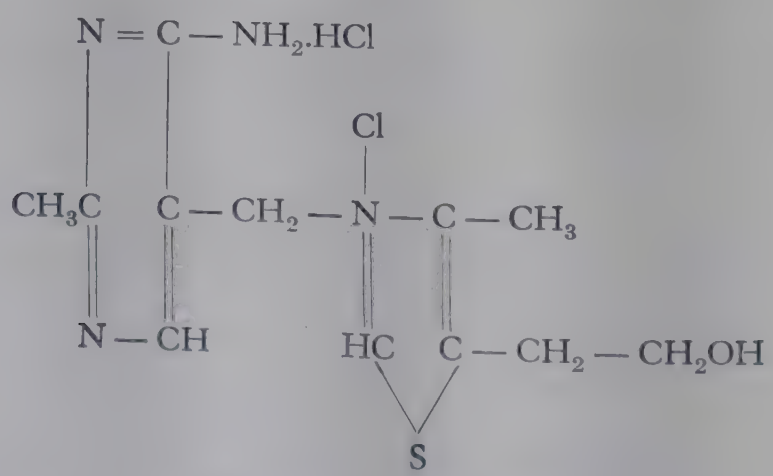
This change in the epithelium is associated with a marked lowering of the local

resistance to the penetration of microörganisms into the tissues, and the patients become peculiarly susceptible to respiratory, urinary tract, and other infections. It is still controversial whether there is in addition a lowering of the general defensive forces of the body.

In experimental animals vitamin A deficiency often leads to the formation of renal calculi and sometimes deposition of calcium in the renal tubules. There is, however, no direct evidence that this is concerned in the formation of urinary calculi in man. Sterility also occurs in experimental animals and is due to persistence of cornified vaginal cells which interfere with implantation. In young puppies vitamin A deficiency causes degeneration of the spinal cord characterized by demyelinization of the nerve fibers. It has been shown also that vitamin A will neutralize the neurotoxin of ergot. These facts have suggested to some that a deficiency in A plays a part in ergotism, lathyrism, pellagra, and even in pernicious anemia and disseminated sclerosis.

Thiamin

The continued deprivation of thiamin (vitamin B_1) produces in man anorexia, cachexia, fatigue states, muscle pains (peripheral neuritis is questioned in true thiamin deficiency), changes in the central nervous system (Wernicke's disease), and cardiologic disturbances. Weakness in the legs may be of polyneuritic origin (in the case of the pig, cat, and rat the polyneuritis is a pyridoxin deficiency disease) or it may be muscle weakness, tenderness, and associated footdrop. Beriberi must be regarded as a constitutional disease with changes in carbohydrate metabolism and weakness; the concomitant lesions may be secondary in character to biochemic abnormalities. The disease may be precipitated in severe form when high caloric diets are introduced or when certain other specific food factors are given.



Thiamin hydrochloride

A chronic deprivation of thiamin in man and experimental animals produces cardiac disturbances. There is a dilatation of the right side of the heart which responds dramatically to thiamin administration. This does not mean that all patients with right-sided heart failure should be given therapeutic doses of thiamin but only those in whom clinical evidence of beriberi exists.

The average daily intake of thiamin seems to be fairly well established on the basis of human experimentation. A constant intake of 0.4 mg. or more does not lead to deficiency symptoms. Below this level in certain individuals symptoms have occurred, but in others they did not manifest themselves (intestinal bacterial synthesis) until sulfaguanidine was administered. However, it may be wise to recommend more than this amount daily, since diets fluctuate considerably and 0.4 to 1.0 mg. of thiamin would obviously be adequate. Therapeutically in beriberi 20 to 50 mg. daily is followed by prompt and dramatic relief. It is at once noted that many foods are rich in thiamin, and the average diet usually contains an excess. The following values are given per ounce and are approximate since the same foods have a tendency to vary considerably. One mg. of thiamin is equal to 333 International Units. Thus 10 ounces of meat contain more than the daily requirements.

Sources:

	International Units
Liver, beef, per ounce	48
Milk, malted " "	50
Butter " "	12
Peanuts " "	60
Wheat germ " "	203
Yeast " "	42 to 420
Almonds " "	24

Clinical Picture in Avitaminosis B_1. The period of development of symptoms is three months or more after the beginning of the inadequate diet and varies markedly with different individuals. There are two clinical types of beriberi: (1) dry and paraplegic and (2) wet or dropsical, but this distinction is an artificial one, for many of the cases present features of both types during the course of the disease. In the former type the patient complains first of muscular weakness and numbness, especially in the legs, and of a sense of fullness and tenderness in the epigastrium. Cardiac palpitation and dyspnea follow the least exertion. The patellar reflex is at first hyperactive, but disappears within a week or two. Hyperesthesia of the calf muscles and blunting of sensation in the hands and feet become noticeable. There is frequently edema over the tibiae and patchy areas of edema may occur anywhere in the body. Later footdrop, wristdrop, general muscular atrophy, and paralysis may appear. Paraplegic patients frequently show the characteristic tripod gait. Leaning forward on a cane with the legs apart, they resemble a tripod. With each step the feet are lifted high and swung outward to avoid scraping the toes. These patients may show a positive reaction to the so-called "jongkok" test: inability to rise from a squatting position with the hands held over the head. In severe cases the paralysis may extend to the diaphragm and intercostal muscles.

The wet type of beriberi is characterized by a marked dilatation of the heart, particularly of the right side, and general edema and effusions into the serous cavities. The blood pressure is always low, particularly the diastolic pressure, with a

corresponding increase in the pulse pressure. Tachycardia occurs after the slightest exertion. The normal rhythm of the heart is replaced by the equal spacing of embryocardia, and systolic murmurs, due to incompetency of the valves, especially the tricuspid, are audible. Sudden death from failure of the right side of the heart is frequent in these cases and may occur in any type of beriberi. This is commonly called "shoshin" and may occur within a few hours after the onset of symptoms. Vomiting is uncommon, but when it occurs, the prognosis is bad.

Minot et al. (1933) have advanced evidence that a deficiency in vitamin B_1 is the cause of alcoholic polyneuritis, either from a limited intake of food or a lack of absorption by a damaged gastrointestinal tract. They report cure of the neuritis by the administration of large amounts of B complex orally and parenterally, even in patients who continue to receive the quantity of alcohol to which they were accustomed.

Excessive amounts of carbohydrate or fat in the diet increase the need for B_1 and may result in the development of symptoms of beriberi.

Ship beriberi is a disease resembling typical beriberi, which was prevalent among the crews of ships on long voyages. It was particularly common on the Scandinavian ships. The main features are edema of the legs, dyspnea, cardiac palpitation, and occasionally death from dilatation of the heart. The chief difference between the two diseases lies in the absence of any manifestations of peripheral neuritis. Nocht noted sore gums and hemorrhages in the muscles in a few cases and considers that the disease is closely related to scurvy, particularly to the condition known as Rand scurvy.

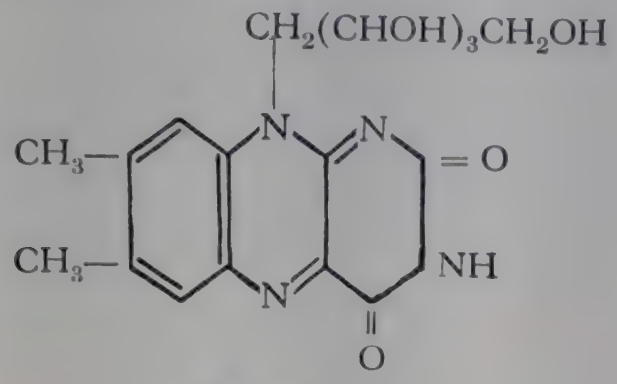
It is quite probable that ship beriberi and other atypical forms of beriberi may be due to diets which are deficient not only in the antineuritic vitamin but also in other vitamins and perhaps in essential minerals.

Riboflavin

Although riboflavin deficiency exhibits fairly clear-cut findings in experimental animals, the lesions in man are not well defined. In the rat there is a marked proliferation of the circumcorneal vessels, and changes in the skin and hair; growth is affected. In the monkey the circumcorneal vessels are not modified particularly, a patchy dermatitis results instead. It may be that the symptoms and lesions vary somewhat in different animal species. It is quite true that the occasional proliferative vascularitis of the cornea seen in man rarely responds to riboflavin medication. The lesions may be old ones derived in childhood or they may be due to the effects of sun and wind or all three. Cheilosis of the lip margins is more often due to malocclusion but may in certain cases be associated with ariboflavinosis. *The mechanism by which a vitamin deficiency predisposes a tissue to environmental or internal factors is as yet unknown.*

The exact riboflavin requirements for man are unknown. Evidence from excretion tests (although of no clinical value) would indicate that somewhere between 0.5 to 1 mg. of riboflavin can be utilized on a daily basis.

Eggs, yeast, milk, liver and certain meats are rich sources of riboflavin. Riboflavin (N.N.R.): 1 mg. per 2 ml. of a 10 per cent urea solution; tablets 1 and 5 mg.



Riboflavin

Niacin (Nicotinic Acid)

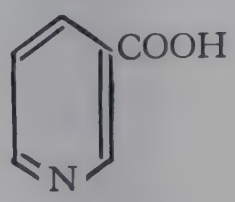
Niacin deficiency induces the fairly well-recognized symptom complex, pellagra. In the experimental animal, niacin deficiency produces black tongue, dermatitis, and gastrointestinal lesions in dogs. Its biochemic function is probably that of cozymase, a part of an oxidation enzyme. In man early clinicians recognized the three "Ds" of pellagra: dermatitis, diarrhea, and dementia. In advanced cases the combination of the three major symptoms is often found. However, today dermatitis and stomatitis are the more common findings.

The most characteristic feature is the skin eruption. Symmetrical scaly patches of erythema resembling sunburn appear suddenly on the most exposed (or irritated) parts of the skin. The commonest sites are the backs of the hands and wrists, often with a narrow band extending across the palmar surface of the wrist joint; the bridge of the nose; the neck, where the eruption often resembles a collar; and occasionally on the dorsum of the feet, and on the scrotum or female genitals. Exposure to sunlight aggravates the burning and itching and sometimes seems to precipitate the outbreak. The patches are erythematous in Caucasians, blackish or purplish in Negroes, and sepia in the olive-skinned races. In severe cases there may be vesiculation and the development of a moist eczematous condition. Petechiae on the affected parts are common. The eruption usually subsides after about a fortnight.

In rare cases the disease runs an acute course and death may occur within a few weeks. Usually symptoms abate in two or three months, but recur the next spring in a more severe form. If not treated this may continue for 10 or 15 years before death ensues. In such chronic cases emaciation becomes extreme. Serious psychoses may develop, characterized by depression, a tendency to suicide, delusions, confusion, and general mental deterioration. Combined sclerosis of the spinal cord is common, with ataxia, spasticity, weakness or paralysis, and incontinence. There is usually a subacidity or achlorhydria and often an anemia, usually hypochromic in type.

The prognosis is good, except for the aged, if the patient is treated early, before the central nervous system is involved. It is unfavorable in very acute, stupor-

ous, or advanced cases. Usually in pellagra there is a deficiency of other vitamins as well. In fact there appears to be evidence that pellagra manifestations may be due to the lack of specific substances other than niacin and that although niacin deprivation may be responsible for inducing some of the major pathologic changes, its absence also induces tissue biochemic changes wherein a more complex general avitaminosis alters the end result. Pellagrins often respond to diets of milk and eggs (not especially high in niacin) if they are at bed rest; it is best to treat the pellagrin not only with niacin but with other members of the B complex and with adequate nutrition.



Niacin (nicotinic acid)

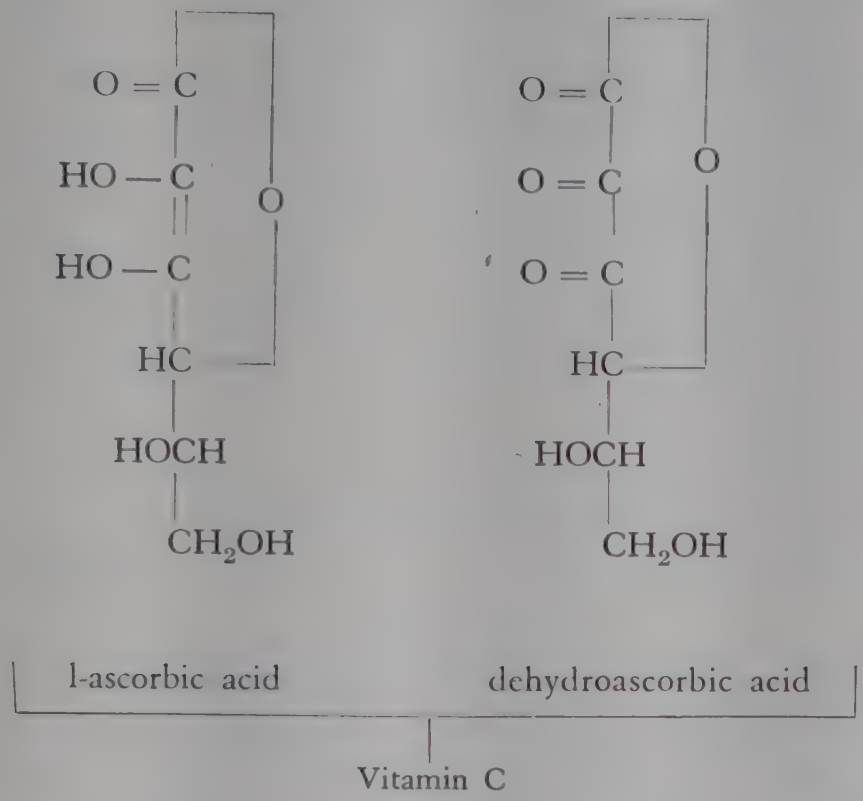
There is some experimental evidence with dogs that diet may influence niacin requirements, and the frequent association of pellagra with malaria has suggested that this disease may alter niacin needs. The dramatic response of pellagrin dementia to niacin has led to the misuse of the vitamin in nonpellagrin dementia. Both tropical and nontropical sprue are somewhat benefited by niacin, but complete recovery requires liver extract or folic acid. The presumed amount of niacin required daily by the human subject is from 10 to 15 mg. In the treatment of niacin deficiency disease 50 mg. can be given, orally, 5 to 6 times daily. It is available in solution (35 mg. in 5 ml.) and in tablets (10 mg.). Meat, pulses, butter, certain vegetables and fruits are rich in niacin.

Vitamin C (Ascorbic Acid and Dehydroascorbic Acid)

Vitamin C deficiency results in scurvy. This disease is characterized by defective collagen formation (intercellular cementum) with weakening of tissue structures. The defective walls of capillaries and the larger blood vessels succumb easily to trauma and bleeding occurs. Thus the disease is characterized by hemorrhagic processes. The first symptoms of scurvy can be characterized by a prodromal period of a few weeks of lassitude, fatigue, and irritability followed by perifollicular hemorrhages, then more generalized ones involving periosteum, joints, and viscera. The hemorrhages of periosteum and joints occur chiefly in those areas which are subjected to greater stress and strain or are easily bruised. The hemorrhages of the viscera occur chiefly in those portions of the bowel which are most active. For some peculiar reason the adrenals are hemorrhagic and swollen. Late in the disease the gums become swollen and hemorrhagic. The appearance of gum lesions is dependent on trauma induced by mastication and a variety of accessory factors.

The maintenance dose of vitamin C to protect human beings against scurvy is between 18 and 25 mg. of the vitamin daily. If large quantities of the vitamin are

consumed, however, a period of protection of from four to five months occurs if the subject is on a vitamin C-free diet. Thus sporadic large intakes followed by intermittent intakes are sufficient to prevent scurvy. No condition other than scurvy can be associated with vitamin C deficiency, and excess amounts of the vitamin do not increase wound healing in the nonscorbutic subject. In the scorbutic subject 1 Gm. of the vitamin should be given daily in divided doses.



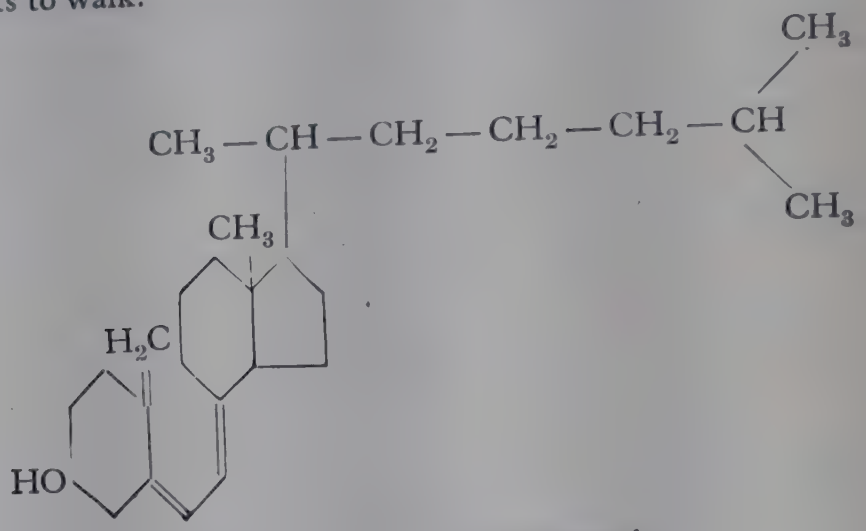
Vitamin D

The fat-soluble vitamin D is concerned with calcium and phosphorus metabolism and acid-base equilibrium. A deficiency of vitamin D is characterized by rickets in the growing animal; poor growth results, osteoporosis manifests itself, and bony changes occur. Vitamin D deficiency in adults is relatively unknown and the process in this stage is poorly understood. Osteoporosis may result from steatorrhea but the mechanism is uncertain. At any rate, large doses of vitamin D seem beneficial in nutritional as well as in war osteomalacia. Increased calcification has been reported in old people on high intakes of calcium and vitamin D, and it may be that the vitamin has pharmacologic effects beyond its ordinary vitamin activity.

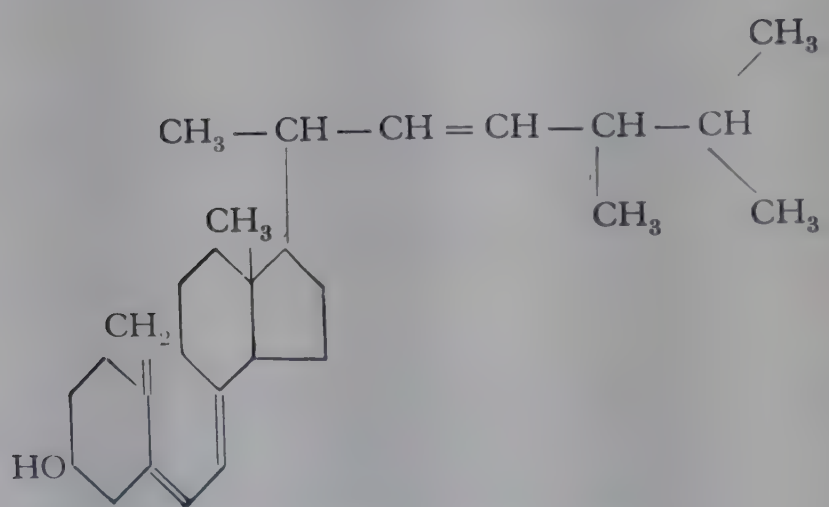
The exact human adult requirements of vitamin D are unknown but it is assumed that some is needed. In the growing child the need for the vitamin is a real one, for if it is deprived, rickets with rachitic rosary, craniotabes, bowlegs, spasmophilia, infantile tetany, osteomalacia, osteoporosis, and dental caries may result.

Bone Changes in Avitaminosis D. The skull and the ribs are the first bony structures to show changes. During the fourth and fifth months the flat bones of the skull are thin and soft (craniotabes); later they show irregular thickenings

with a characteristic protuberance of the frontal and parietal bones. The fontanelles are slow to close. The ribs become enlarged at the costochondral junction (the rachitic rosary) and are depressed at the attachment of the diaphragm. Thickening of the epiphyses proximal to the wrists and ankles is very characteristic. The legs are apt to become deformed—bowlegs, knockknees, saber-shins, etc.—after the child starts to walk.



activated 7-dehydrocholesterol



activated ergosterol or calciferol

The affected bones show characteristic chemical and anatomic changes. The inorganic matter, as measured by the total ash content, is greatly reduced, even to 50 per cent in severe cases. Even when the reduction in the total quantity of calcium and phosphorus is marked, however, the ratio between them remains the same as in normal bone. The histologic picture is characteristic. The zone of provisional calcification at the junction of the epiphysis and diaphysis is absent, and the zone of proliferative cartilage is wide and irregular (the rachitic metaphysis). Blood vessels from the shaft penetrate into the latter zone and absorb the cartilage in scattered spots, and the orderly arrangement of the cartilage cells is completely destroyed. There is a compensatory overgrowth of uncalcified osteoid tissue in masses along the shaft and at the ends of the bones, giving rise to the visible enlargements. These changes are pathognomonic of rickets, and their

demonstration by roentgenograms is an important point in the diagnosis of early cases. During convalescence the histologic picture returns to normal, and calcium salts are gradually deposited in an even, narrow line in the zone of provisional calcification. The McCollum-Shipley "line test" used in the assay of vitamin preparations is based upon the appearance of this zone after immersion of a longitudinal section of the bone in a solution of silver nitrate. The smallest amount of the unknown preparation which will produce a normal line in a rachitic rat within a given period (10 days) is taken as 1 unit.

Treatment. However, vitamin D dosage should be controlled, since excessive amounts result in pathologic calcification and other toxic effects. In fact, the infant dosage should not exceed 600 units daily since recent reports indicate difficulties when 1800 units are administered on a daily basis. It is well established that 400 units daily will prevent rickets. In treating rickets, however, large doses are initially required, even 100,000 units or more.

Average daily requirements as recommended:

	U.S.P. Units
Infants	400 to 600
Children	300 to 600
Adults	300 to 600
Lactation pregnancy	600 to 800

Preparations:

	U.S.P. Units per Gm.
Cod liver oil (U.S.P.) preparations vary	85 to 350
Halibut liver oil (N.N.R.)	8,500
Activated ergosterol (Viosterol)	10,000

Rickets. Rickets is caused by a disturbance of the metabolism of calcium and phosphorus due to a deficiency of vitamin D. Ca, P, vitamin D, and parathyroid hormone are all essential for the mineralization of normal bone, but the exact part played by each and their precise relationships to one another are not known. The action of parathyroid hormone in maintaining the level of calcium and phosphorus in the blood has been discussed in the chapters on blood chemistry. It is generally believed that vitamin D acts by promoting the absorption of calcium and phosphorus or by regulating their excretion by the intestinal tract and kidney. The development of the disease depends also to some extent upon the ratio of calcium to phosphorus in the diet. If the phosphorus intake is low, absorption is blocked by the formation of calcium phosphate in the intestine (provided the supply of vitamin D is limited), and the disease can be cured in some cases simply by restoring the normal balance between them (two parts of calcium to one of phosphorus).

Some authorities believe that vitamin D acts by stimulating the parathyroids. Compensatory hyperplasia of the parathyroids has been described in rickets and also in osteomalacia, and Hamilton and Schwartz have reported an excess of parathyroid hormone in the blood of rachitic infants. The blood phosphatase is greatly increased.

In most cases of rickets the phosphorus content of the blood is reduced (below 3 mg.) and the calcium is approximately normal. Less frequently the calcium is reduced and the phosphorus normal, the so-called "low calcium rickets." If either element is deficient in the blood, normal deposition of calcium phosphate in the osteoid tissue does not occur. Howland has pointed out that when the product of the calcium content of the blood in milligrams multiplied by that of the phosphorus, the "solubility product constant," is below 30, rickets is invariably present, and it is usually present when the figure is 40 or less. The low calcium cases may be complicated by tetany. The amount of calcium and phosphorus in the feces is high, and that excreted in the urine is low.

Administration of vitamin D restores the quantity of calcium and phosphorus in the blood to normal and increases the amount excreted in the urine. It relieves tetany, if this is present. Tetany is also relieved by the administration of parathyroid hormone, but this does not cure the rickets.

Vitamin K

Vitamin K at the present moment finds its sole therapeutic use in the treatment and prevention of hypoprothrombinemia. A suggested classification of the clinical conditions in which hypoprothrombinemia in man may occur is therefore presented in Table 82. It will be observed that three mechanisms exist by which prothrombin deficiency may be produced: (1) nutritional deficiency of vitamin K, lack of production by the intestinal bacterial flora, and failure of absorption of the vitamin; (2) failure of production of prothrombin by the liver; and (3) increased destruction or utilization of prothrombin.

The hypoprothrombinemia of hemorrhagic disease of the newborn is in all probability consequent upon the initial sterility of the gastrointestinal tract. This hemorrhagic disorder has now been successfully treated with vitamin K and prevented by the administration of this factor. It is of interest to note that Castle in 1938, correlating the observation that hemorrhagic disease of the newborn was due to a prothrombin deficiency and the observations of Dam, correctly predicted the eventual successful treatment of this disorder with vitamin K.

Not infrequently two of the mechanisms leading to hypoprothrombinemia may operate simultaneously, as in extrahepatic biliary obstruction and secondary liver injury. Of the defects leading to hypoprothrombinemia presented in Table 82 the most amenable to vitamin K therapy are those in the first main classification: namely, those due to dietary deficiency, failure of manufacture by intestinal bacterial flora, and failure of absorption of this factor.

The exact diagnosis of hypoprothrombinemia cannot be made without the demonstration of a diminished prothrombin concentration in the blood by laboratory means. Several methods for this determination have been devised, and it is not intended to enter into the controversy concerning the respective merits of these methods.

Sources. There are two main sources of vitamin K for human needs, an extrinsic source from the diet, most abundant in chlorophyll-containing plants,

Table 82

A Classification of Hypoprothrombinemia in Man (Lozner and Kark)

A. Nutritional Deficiency of Vitamin K
  1. Dietary Deficiency
    (a) Occasional patients with scurvy and pellagra
  2. Failure of Manufacture of Vitamin K by Intestinal Bacterial Flora
    (a) Hemorrhagic disease of newborn
    (b) Various intestinal disorders [See A-3(b)]
  3. Failure of Absorption of Vitamin K
    (a) Due to exclusion of bile from intestine
      (1) Biliary obstruction
      (2) Biliary fistula
    (b) Various intestinal disorders
      (1) Nontropical sprue
      (2) Ulcerative colitis
      (3) Polyposis
      (4) Regional ileitis
      (5) Fistulae and obstructions
B. Failure of Production of Prothrombin by Liver
  1. Acute or Subacute Necrosis of the Liver
  2. Cirrhosis of the Liver
  3. Liver Injury Secondary to Extrahepatic Biliary Obstruction
C. Increased Destruction or Utilization of Prothrombin
  1. Hyperpyrexia
  2. Certain Surgical Operations, Particularly Those on Biliary Tract

and an intrinsic one derived from the bacterial flora of the gastrointestinal tract. Dam considers the latter source the more important for human needs. The vitamin K from both plant and bacterial sources is fat-soluble, requiring the presence of bile salts for absorption. Recently the chemical structure of both forms has been determined and both have been synthesized. Both are naphthoquinone derivatives. Of great interest and significance has been the discovery that certain synthetic analogues of natural vitamin K, particularly 2-methyl-1, 4-naphthoquinone, possess even greater physiologic activity than does the natural material. Certain of these are water-soluble and therefore may be administered by mouth without bile salts, or parenterally.

An Outline of Vitamin K Therapy

A. Preparations Available
  1. Oral
    (a) Synthetic analogues
      (1) 2-methyl-1, 4-naphthoquinone
    (b) Natural concentrates
      (1) Alfalfa
      (2) Cereal plants
  2. Parenteral
    (a) Water-soluble synthetic analogues
      (1) 2-methyl-1, 4-naphthoquinone-3-sodium sulfonate
      (2) 4-amino-2-methyl-naphthol hydrochloride

B. Indications
   1. Existent Hypoprothrombinemia
      (a) Bile present in intestinal tract: oral preparations
      (b) Bile absent from intestinal tract: oral preparations plus bile salts
      (c) Vomiting, severe intestinal disease, surgical emergency or failure of response to oral therapy: parenteral preparations
   2. Latent Hypoprothrombinemia
      (a), (b), and (c). As above
   3. Last week of pregnancy: oral preparations

C. Dosage
   1. Treatment of existent hypoprothrombinemia
      (a) Synthetic analogues: 2 to 6 mg. daily
      (b) Natural concentrates: 2 to 4 Gm. daily
   2. Treatment of latent hypoprothrombinemia
      (a) Synthetic analogues: 1 to 2 mg. daily
   3. Last week of pregnancy
      (a) Synthetic analogues: 1 to 2 mg. daily

D. Toxicity
   1. Synthetic analogues
      (a) 2-methyl-1, 4-naphthoquinone
         (1) 180 mg. by mouth are the smallest dose that has caused vomiting and porphyrinuria in adult human beings
         (2) 0.5 Gm. per kilogram by mouth is minimal lethal dose in mice
   2. Natural concentrates
      No toxicity reported

Vitamin E

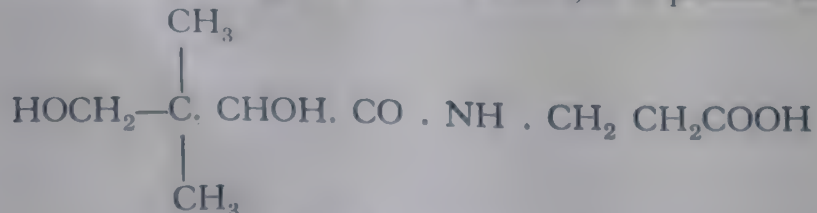
Vitamin E is known as the antisterility vitamin. Absence of this vitamin induces habitual and threatened abortion in cattle. Little is known of this vitamin, and there is no clinical evidence for its effectiveness in man.

Sources: Wheat germ oil; cottonseed oil; rice oil; lettuce; whole grain cereals.

Preparations: Not included in U.S.P. or N.N.R.

Pantothenic Acid (Vitamin B_3)

Since the symptoms of pantothenic acid deficiency in man are unknown, the subject will be briefly discussed. The vitamin appears to be necessary for rats and birds. Its lack causes incrustations about the eyes, the corners of the mouth, and the areas between the toes. The skin epithelium becomes keratinized and a dry slough occurs. Besides dermatitis certain lesions are found in the spinal cord, characterized by the degeneration of myelinated fibers. In rats and mice deprived of the vitamin, a retardation of growth occurs and a symmetrical depigmentation of the fur takes place. This process has been observed also in foxes. Rats develop the so-called "spectacled eye condition." The vitamin occurs in all types of animal tissues, the liver and kidney being the richest sources; it is present in most plants.

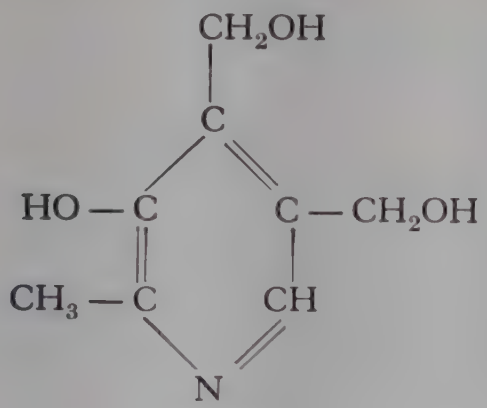


Pantothenic acid

No International Unit for pantothenic acid has been established, but the yeast unit appears to be the most satisfactory. According to this test, the increase in growth of yeast following the administration of pantothenic acid is determined (Williams and Saunders).

Pyridoxin, Vitamin B_6 of György

Vitamin B_6-avitaminosis in rats causes a specific symmetrical dermatitis called acrodynia, which affects the peripheral parts of the body, such as the paws, the mouth, the tail, the ears, and the nose, and is accompanied by edema and scaliness. In dogs, rats, and pigs subnormal growth and epileptiform seizures occur as well as the dermatitis. The present-day knowledge of vitamin B_6 in human beings is limited, and the necessity for a dietary source of the vitamin in man has not been proved. So far large doses of the vitamin in man appear to induce a sedative effect.

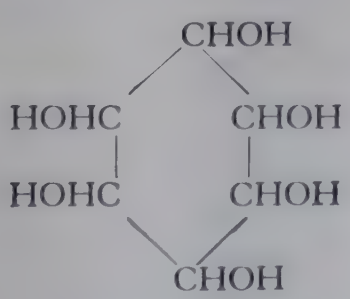


Pyridoxin
1, 3-hydroxy-4, 5-di(hydroxy-methyl)-2 methyl pyridine

Vitamin B_6 is widely distributed over the entire animal and plant kingdom and is chemically bound to protein and starch. The method of assay is microbiologic, using either yeast, bacteria, or rat growth as an index of quantity. The growth and production of acid by *Streptobacterium plantarum* corresponds to the amount of pyridoxin present. One rat unit of pyridoxin, 10γ, is defined as the amount necessary per day to cure or prevent typical symptoms of avitaminosis (György).

Inositol

Manifestations of inositol deficiency occur in young mice and rats. These animals require inositol for normal growth and maintenance of hair. Thus white mice on an



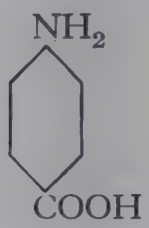
Inositol (bios I)

inositol-free diet become completely hairless, and a severe dermatitis results. A deficiency also leads to the development of fatty livers and cholesterol-induced type of cirrhosis. The role of inositol in human economy is unknown.

The vitamin occurs as a normal cell constituent of practically all plant and animal tissues; yeasts, molds, and bacteria contain it.

Para-amino-benzoic Acid (Growth Factor P for Bacteria)

In the animal organism *p*-amino benzoic acid counteracts the action of hydroquinone, which, for example, in the cat and in mice causes a graying of the fur. The action of sulfonamides in infected mice is also inhibited by the acid. It has a pronounced influence on tyrosinase activity, inhibits the oxidative destruction of adrenalin, and blocks the action of certain enzymes. A deficiency of *p*-amino benzoic acid causes a graying of fur in the black or piebald rat, a syndrome called "nutritional achromatrichia." A retardation of growth occurs in the chick. The acid is required as a growth factor for many bacteria, and the presence of this vitamin is required in most culture media.



Para-amino-benzoic acid

The vitamin is widely distributed in most plant and animal tissues, and it is a natural constituent of yeast where it is present as a benzoyl derivative. The role of the vitamin in human economy is as yet not ascertained.

Unidentified Vitamins

The existence of a special factor (vitamin B_4) which prevents the occurrence of paralysis in rats and chicks has been postulated. The vitamin is present in high concentration in yeast, liver, dried grass, wheat germ, pork brain, and pork kidney.

Vitamin B_5. This is probably identical with nicotinic acid since the eluate containing the fraction exhibits all of the biologic effects of the latter. The vitamin is essential for maintaining weight in pigeons, a phenomenon produced by nicotinic acid as well.

Vitamin B_7 (Or vitamin I). Vitamin B_7 is a substance present in rice polishings, soluble in methyl and ethyl alcohol, needed by pigeons for protection against specific digestive disturbances.

Adenylic Acid (Vitamin B_8). This vitamin appears to be necessary for certain strains of lactic acid bacteria. In pellagrins the acid seems to increase the effectiveness of nicotinic acid.

Vitamin J. If guinea pigs are fed lemon juice, they show a remarkable resistance to pneumococcal infection. The factor is not vitamin C, since the effect is not obtained by synthetic vitamin C, paprika, or eye-lens concentrates. The factor for want of a more definite term is denoted as "vitamin J."

Vitamins L_1 and L_2. There is postulated the presence of vitamins L_1 and L_2, necessary for normal lactation. The richest source of these vitamins is in liver and yeast. Neither factor can replace the other, but both are necessary for the maturation of lactation tissue.

Vitamin M. Vitamin M is chiefly found in liver but occurs in other tissues. Monkeys on a diet containing all of the other dietary factors but lacking this particular fraction develop anemia, cytopenia, loss of weight, ulceration of the gums and diarrhea. Its absence results in decreased resistance to dysentery organisms.

Factor T. Factor T, occurring in sesame oil and egg yolk but not in cod liver oil or

olive oil, destroyed by ultraviolet irradiation, protects against thrombocytopenia in man and rats. The absence of the factor results in a decrease of blood platelets.

Folic Acid. In 1941 Mitchell, Snell, and R. J. Williams reported the separation of a concentrate from spinach to which they gave the name "folic acid" (derived from the Latin *folium—leaf*). It was the active principle for the growth of *Streptococcus lactis* R., under specified conditions. It has just recently been crystallized but there is a definite indication today that sources of folic acid assayed microbiologically may contain more than one nutrilite since the factor which stimulates the growth of *S. lactis* may be different from that which acts on *L. casei*. Thus Snell and Peterson postulated what has come to be called the *L. casei* eluate factor. A comparison of the eluate factor from different sources reveals the following potencies:

	S. lactis	*L. casei*
Solubilized liver	100	100
Whole liver substance	186	68
Grass juice powder	147	68
Dried grass juice powder	11	29

There are further biologic differences between the factors which indicate that *L. casei* eluate factor and so-called folic acid are not identical. Recently Briggs and his associates have reported two vitamins B_{10} and B_{11} necessary for the feathering of birds and the growth of chicks and not replaceable by *L. casei* factor although having certain properties in common with reference to bacterial stimulation. The known *L. casei* eluate factor requirements are as follows:

(a) Bacteria for growth: *L. casei, Lactobacillus helviticus, Profionbacterium pentoseaceum, S. lactis* R., Tetanus bacillus, Tetanus toxin.

(b) Chicks: for growth and anemia prevention.

(c) Rats: for growth.

(d) Guinea pigs: for growth.

(e) Monkeys: for prevention of leukopenia and anemia.

(f) Dogs: for prevention of normocytic anemia.

(g) Relation to human needs unevaluated.

Considerable experimental work is under way today to determine the role of folic factors in animal economy. For instance, there is some evidence to show that this factor can obviate sulfonamide anemia in rats and, as such, suggests that certain sulfonamides destroy gastrointestinal bacteria involved in the synthesis of this vitamin.

Since rats with folic acid deficiency develop agranulocytosis and in some cases anemia which is cured by the administration of *folic acid*, the agent has been tried experimentally in patients with sprue and pernicious anemia. The results so far reveal a remission of the disease in sprue and a marked reticulocyte and hemoglobin increase in pernicious anemia, although the neurologic disturbances do not invariably improve. (See p. 439.) The vitamin, furthermore, may interfere with glutamic acid metabolisim in pernicious anemia patients.

Folic acid has been found to be intimately involved in the nucleic acid

metabolism of bacteria. The substitution of small amounts of the pyrimidine, thymine (5-metoyluracil), satisfies the need of bacteria for folic acid. The compound is so insoluble, however, that large amounts are needed in man to yield the results obtained with folic acid in sprue. More recently introduced water-soluble thymine compounds (glucosyl thymine) show but little promise in early experimental tests.

Factor U. Factor U is a growth promoting substance required by chicks and is present in yeast, wheat bran, middlings, alfalfa meal, and corn.

Vitamin B_c. Vitamin B_c occurring in liver appears to be necessary for the chick with regard to prevention of decreased erythrocyte count, decreased hemoglobin, and decreased red-cell volume.

Vitamin B_p (Antiperosis Vitamin). Vitamin B_p is a vitamin present in nonsynthetic diets protecting chicks against hock disease or slipped tendon, twisting and shortening of bones.

Special Factor in Fresh Grass. The existence of a special factor in fresh grass appears to be necessary for the growth of rats and guinea pigs. It is found in all fresh greens but is present only in small quantities in animal tissues such as liver and kidney. The factor appears to be different from all other known factors and can be precipitated and removed with acetone from grass juice. It is readily absorbed by animals and is secreted into milk. Therefore summer milk of cows contains considerable quantities whereas winter milk is practically devoid of it.

Biotin. Bios III or Miller's bios IIb, adsorbable on charcoal, was shown by Kogl to contain a powerful growth stimulant to which he gave the name "biotin." It was isolated from duck egg yolk but can be extracted from molasses, hydrolyzed yeast, commercial sucrose, and other substances. In 1927 Boas described a syndrome in rats on a diet containing dried raw egg white as a source of protein. The syndrome failed to occur when potatoes, milk, or liver were added to the diet, and she postulated the presence of some protective factor in these substances. She interpreted her evidence to indicate that raw egg white contained a toxic substance which could be neutralized by a protective vitamin. In 1940 Williams and his associates produced evidence that the substance that caused egg-white injury was united with biotin in a fairly stable combination but was decomposed by hydrolysis. The factor in egg white was a protein to which was given the name "avidin" because of its avidity for biotin. The presence of biotin in intestinal contents has been shown to be the result of synthesis by intestinal bacteria, and if avidin is given in sufficient amounts it will combine fully with biotin, and the result will be egg-white injury disease. Biotin is one of the most powerful growth stimulants yet discovered, 0.00004γ will cause a 100 per cent increase in growth of microörganisms. The universal distribution of biotin and its presence in quantity in seeds suggest that it may have a very general function.

Dehydration and Electrolyte Depletion

It is not possible to discuss water losses without reference to electrolyte changes, nor to consider shifts in electrolytes without awareness of resulting changes in body fluids. Water conservation is of the utmost importance as a loss of only 6 per cent of body water will be accompanied by severe symptoms and signs.

The water requirements of the body are governed by body weight and surface area, temperature and humidity of the environment, and exercise. The actual volume of water required as such is further determined by the food intake, or in the absence of it by the nature of the body tissues metabolized to furnish necessary calories. Thus, the minimum water required by an individual to prevent dehydration can be calculated if the above factors are known. The figure so arrived at is not the minimum compatible with preservation of life over short periods but that required to maintain water economy at usual levels. For short periods the quantities may be significantly less. To provide for renal and basal extrarenal water loss, a weight of water is necessary which is numerically equal in grams to the individual's surface area in square meters multiplied by the factor 650. The average person has a surface area of about 1.7 square meters and thus would require a minimum of 1100 ml. of water per day. Exercise will increase the need for water, and this need can be calculated in grams of water as it is numerically equal to 0.702 times the exercise requirement in large calories. These derivations are empirical but rest upon sound concepts. Part of the water requirements can be met through the metabolism of food or tissue. Per 100 Gm. of foodstuff metabolized, fat yields 107 Gm. of water, carbohydrate 55 Gm. and protein 41 Gm. This is the water of metabolism of the pure dry foodstuffs and not the naturally occurring foods as usually taken since these contain large quantities of free water in addition. Metabolism of protein increases water requirements by the amount necessary to provide for the electrolytes which are liberated in protein oxidation and to provide a vehicle for the excretion of nitrogenous residues. The water actually available from protein metabolism is thus somewhat less than the above figure. No water is required for the disposal of end products of metabolism of fat and carbohydrate; hence, the water resulting from their oxidation is all available for metabolic needs. The end results of oxidation insofar as water requirements are concerned are the same regardless of whether the foodstuff metabolized is ingested or is derived from body tissues.

It is thus evident that measures aimed at conservation of water in a semifasting man will be concerned primarily with three factors: (1) exercise, (2) sweating, (3) protein tissue breakdown. Exercise can readily be held to a minimum when the advantages to be gained are not commensurate with the risks involved in so increasing water needs. Sweating as a temperature control mechanism can be kept at a minimum by keeping body surfaces moist with sea water and avoiding direct sunlight. Destruction of body protein can be held to a minimum by taking advantage of the protein-sparing action of fat and carbohydrate and providing this type of foodstuffs in emergency rations.

It is of interest that such calculated water requirements and provision for water conservation accurately predict recommendations made following actual trials under field conditions. Futcher, Consolazio, and Pace[12] found that 500 ml. of water per day were sufficient to maintain men on pneumatic life rafts in the Gulf of Mexico. Gamble and Butler[13] have shown that the renal requirement for water can be reduced by one-third to one-half by glucose ingestion which not only spared

tissue protein but also reduced sodium chloride losses in the urine. Butler's recommended lifeboat ration of a candy containing 80 per cent glucose and 20 per cent fat provides 5 calories and 0.66 Gm. of water per gram of ration. If sufficient calories to maintain life are taken, this diet will almost provide enough water for minimal requirements exclusive of that required for temperature control through sweating and that for exercise.

It appears that the above measures should be relied upon to maintain life in castaways. The use of fish juices is practical only to augment the fresh water supply as Butler was able to obtain only 50 ml. of fluid by prolonged mechanical squeezing of 1 kilogram of sea bass. To provide 500 ml. of fluid in this manner would require extraction of tissue juices from 22 pounds of fish.

On a water restricted regimen, ingestion of salts or hypertonic solution such as sea water will very significantly increase water requirements and under these circumstances should be assiduously avoided.

Under ordinary conditions with some effort to hold sweating at a minimum, losses of electrolytes will not affect the viability of a castaway. Over 30 per cent of body sodium can be lost gradually over a period of three weeks without seriously incapacitating a man. Electrolyte equilibrium can be satisfactorily protected by adding one part of sea water to four parts of fresh water and drinking such a mixture one day in each four. If desalinated water is used no sea water should be added as this water contains sufficient electrolytes to prevent significant losses.

The use of salt tablets to supplement dietary salt for men sweating heavily must be recommended only with appreciation of the individual's dietary intake and his degree of acclimatization. Indiscriminate use of salt tablets is deprecated, but when clear indications for its use exist, dosages should be roughly calculated. Total sodium chloride intake for working men sweating heavily in humid climes need not exceed 20 Gm. per day and 15 Gm. will almost always be adequate. The average diet will provide 10 Gm. of salt a day, and slight salting of food readily brings this up to 15 Gm. Enteric coated tablets will largely avoid the nausea frequently encountered following salt administration.

Trauma and Nitrogen Metabolism

The nitrogen economy of the body is peculiarly sensitive to disturbances of body function whether caused by disease or trauma. That nitrogen loss occurs with fever and acute infectious disease has been much more generally recognized than that nitrogen loss follows physical trauma to parts of the body. The excessive loss of nitrogen after burns is perhaps an exception. The mechanism of these losses is not known.

The average normal adult who is receiving adequate calories in his diet can readily be brought into nitrogen equilibrium on an intake as low as 0.4 Gm. of protein per kilogram ideal body weight per day. The generally accepted figure for optimal protein intake for adults is 1.0 Gm. per kilogram ideal body weight per day. In persons with disease and following trauma the minimal nitrogen

intake required to maintain nitrogen equilibrium is much increased, and following injury in some instances in which it has been determined it is so large that balance is very difficult to attain with natural protein-rich foods. About 2.0 Gm. of protein per kilogram ideal body weight per day is the practical limit of a diet which can be taken by an individual without glut of the appetite. Even this level is very difficult to attain in many persons. With such a large increase in the minimal nitrogen intake as occurs in trauma, the optimal level is unknown. It is reasonable to suppose that it is only moderately above the amount required to restore equilibrium as we appear to be dealing with a disturbance of function which in part reverts to normal upon reaching equilibrium.

It has become the custom to look upon the large nitrogen losses following trauma, such as fractures, as not only undesirable but even harmful. Attempts to restore the patients to nitrogen equilibrium have seldom been successful until approximately the second week following injury when nitrogen equilibrium is generally spontaneously restored on normal protein intakes. It must be emphasized that we are discussing nitrogen losses which occur despite ingestion of what would normally be an adequate protein intake. This has no relation to the undesirable effects of low protein diet on wound healing which was recognized by Clark in 1919 and which more recently has been extensively studied by Ravdin and others. We are not primarily concerned with why malnourished patients are poor surgical risks but whether suppression of nitrogen losses in good surgical risks will decrease the risk and significantly shorten convalescence.

The absorption of nitrogen in these patients is unimpaired; the losses are accounted for by a large increase in urinary nitrogen. The same wasting of nitrogen is seen when endogenous protein is the source. The nitrogen loss may be accompanied by other bizarre phenomena during the period of protein catabolism: e.g., Howard[14] noted a retention of potassium equal to about 20 per cent of the total potassium in normal men.

Plasma protein concentration can be used only as an index of severe nitrogen depletion. Weech, Wollstein, and Goetsch showed that for each gram of nitrogen lost from the plasma protein, 5.3 Gm. were lost from hemoglobin and 22.5 Gm. from body tissues. The actual depletion of blood constituents may be masked by concomitant changes in blood volume. The liver is depleted of protein to a greater extent than are other organs, and although with moderate depletion no failure in function has been demonstrated, the reserve capacity must be affected.

Methods of prevention of nitrogen loss are at present confined to feeding increased quantities of protein. In patients with disturbances of the intestinal tract it may be advisable or necessary to feed amino-acid mixtures. These can be given either orally, by tube, or intravenously. The use of testosterone to promote nitrogen assimilation in these patients is of doubtful value.

It is thus apparent that the factors involved in recovery from wounds and fractures are poorly understood in their relation to nitrogen economy. The further study of protein metabolism in relation to convalescence is one of the most challenging problems at present being attacked. However, until the final solution is

attained, one can do much for his patient by careful attention to his protein intake to insure that he is receiving a generous nitrogen intake approximating between one and one and one-half times the optimal intake for the normal individual.

Nutritional Edema (War Dropsy, Famine Edema)

During World War II a condition characterized by weakness and edema was observed in the parts of Europe where the shortage of food was most acute.

In certain countries, such as Haiti and Java, a form of nutritional edema is prevalent among classes whose diet is for any reason inadequate. Among infants who live for long periods on a preponderantly starch diet, striking degrees of dropsy are seen which appear similar to nutritional edema in all respects.

Clinical Picture. The edema is more marked than in ordinary cases of starvation. It is most prominent in the legs and feet. At times it extends to the thighs and trunk and, in about half of the cases, the face. Upon disappearance of the edema, marked emaciation is apparent. Extreme muscular weakness and disturbances of the alimentary tract are common. Slight exertion is followed by dyspnea and a slow pulse, although cardiac disturbance is not a feature of the disease. The urine is pale, with a low specific gravity, and free from albumin. There is an anemia, with a tendency to leukopenia. Ocular manifestations presumed to be due to a deficiency of vitamin A were also noted in Central Europe where there was a shortage of fats.

Etiology. Nutritional edema is generally ascribed to an inadequate and unbalanced diet of low-protein content. Deficiencies in mineral substances and in vitamins may also be important factors. The condition is aggravated by heavy physical work. Chemical studies of the blood show a decrease in the plasma proteins. A similar condition can be produced in animals by diets which are deficient in protein and certain mineral salts.

Treatment. Treatment with high-protein diets may bring about a prompt restoration of the plasma protein with recovery. In some cases, however, the response is slow, possibly from defective absorption or utilization of the protein.

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Appendix

Appendix

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A. Apparatus

The Microscope

The most important piece of apparatus for the laboratory worker is a good compound microscope. It should be equipped with a triple or quadruple nosepiece and a removable mechanical stage. A monocular is adequate for almost all purposes but if long hours are to be spent using the microscope a binocular soon pays back its extra expense in saving to the eyes. Binoculars are almost a necessity in busy hematology, parasitology, and pathology laboratories. A stereoscopic (dissection) microscope is essential in the examination and dissection of entomologic and helminthologic specimens. A simple microscope (magnifying glass, hand lens) is only rarely needed, and an ocular may be substituted when such low magnification is required.

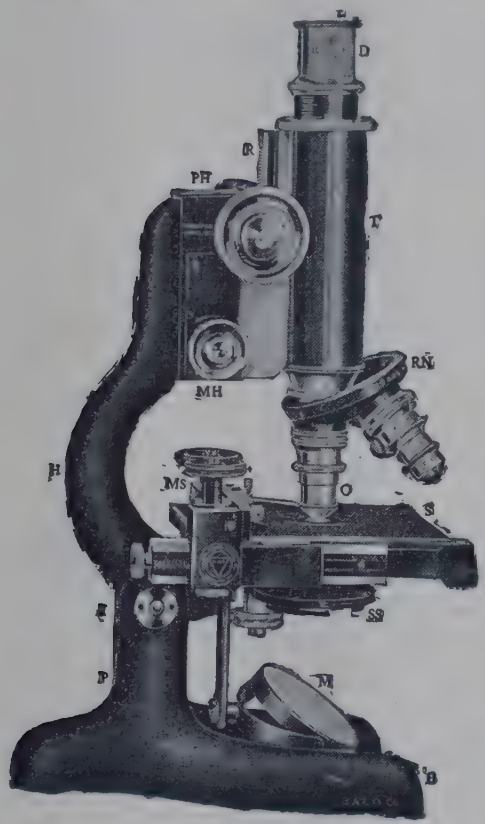
Objectives. These are the most important lenses of the microscope and should be selected with great care. They vary chiefly in color correction, numerical aperture, equivalent focus, and magnification.

Color Correction. Three general types of objectives are manufactured: (1) the achromatic, corrected for two colors and spherically for one; (2) the apochromatic, corrected for three colors and spherically for two; (3) the semiapochromatic (fluorite), whose color correction is better than that of the achromatic but not as good as the apochromatic. Achromatic objectives are adequate for all general work and are much cheaper than the other types. The better lenses are valuable in hematology, bacteriology, malariology, and photomicrography.

Numerical Aperture. Most objectives have the letters N.A. and a figure marked upon them expressing their numerical aperture. From a practical standpoint this gives the relative proportion of the rays which proceeding from an object can enter the lens of the objective and form the image. Of course, the greater the number of rays, the greater the N.A., the better the definition, and consequently the better the objective. The *resolving power* of a lens, i.e., the shortest distance between two lines or points at which they can be distinctly seen as two instead of one indistinct point or line, is closely related to the N.A. The wave length of light divided by the N.A., or twice this figure if a condenser is used, will equal the theoretical resolving power of the correctly employed lens. For example: Using green light (wave length about 0.55μ) and an oil immersion objective (N.A. 1.25) with a condenser, then $0.55 \div 2(1.25) = 0.22\mu$ or the resolving power of the objective.

Equivalent Focus. An objective is usually designated by its equivalent focal distance, i.e., the focus of a simple theoretical lens which has the same power and characteristics as the combination of lenses in the given objective. It does not represent the working distance of an objective, by which is meant the distance from the upper surface of the coverglass to the lower surface of the objective. The usual objectives are the 16-mm. (low dry), the 4-mm. (high dry), and the 2-mm. (oil immersion).

Magnification. Modern objectives are usually marked with their magnifying power. This figure multiplied by the magnification of the ocular used with the lens will give the total magnification of the objective-ocular combination at the eye point. For clinical microscopy, objectives of three powers usually suffice, a low power (10×), a medium power (40×), and a high power (90×). 3×, 20× and 60× lenses may also prove valuable.



Parts of microscope. (E) Eye-piece. (D) Drawtube. (R) Rack (coarse adjustment). (PH) Pinion head. (T) Body tube. (MH) Micrometer head (fine adjustment). (RN) Revolving nose-piece. (O) Objectives. (MS) Mechanical stage. (S) Stage. (H) Handle, a part of the arm. (SS) Substage. (I) Inclination joint. (P) Pillar. (M) Mirror. (B) Base.

Most individuals are able to use a magnification of between 800 and 1000 times the numerical aperture of the objective. This depends upon the resolving power of their eyes. Magnification beyond this resolving power is "empty," i.e., no more details are seen if the magnification is increased by the use of a higher ocular.

Immersion Objectives. Although most objectives are constructed to work in air certain ones of high magnification require the immersion of the front lens in a fluid, usually oil. Several kinds of immersion oil are used, cedarwood oil probably giving the best optical conditions but being sticky and gummy. For general laboratory use mineral oil is to be preferred. A drop of oil should also be placed between the upper surface of the condenser and the lower surface of the slide if maximum definition is to be obtained with an oil-immersion objective. In the clinical laboratory, however, this is seldom required.

Oculars. The ocular in common use is known as a negative or Huygenian lens, i.e., an ocular in which the lower lens (collective) assists in forming the real inverted image which is focused at the level of the diaphragm within the ocular. The negative ocular must be reversed in order to use it as a simple magnifier. The second type of eyepiece, the positive ocular, acts as a simple microscope, magnifying the image formed entirely by the objective and located below the ocular. In order to get full advantage from apochromatic objectives, especially in photomicrography, it is necessary to employ with them so-called compensating oculars which are designed to compensate for and correct the residual color defects in the extra-axial portion of the visual field. These oculars may also be used with the ordinary achromatic objectives.

By fixing one end of a hair on the rim of the diaphragm inside the ocular with a minute drop of balsam one has a satisfactory pointer to locate any particular cell in the microscopic field.

Use of the Microscope. If the microscope is to be used practically and intelligently the following rule must be kept constantly in mind: *The higher the magnification the smaller the field of view, the shorter the working distance, and the less the depth of*

focus. Always use the lowest power practicable as the field is larger and the image sharper. This applies to the ocular as well as to the objective.

All preparations should be examined first with the low-power objective to select suitable areas for further examination and to get all possible details. With tissue sections a preliminary study with a magnifying glass or even the unaided eye may give a surprising amount of information.

Position. Although some workers prefer to use the microscope with the body tube inclined by the inclination joint, yet one gets just as good results by keeping the tube perpendicular and it is better to become accustomed to such a position since it is necessitated when working with fluid mounts.

The Eye. It is advisable to cultivate the use of both eyes in doing microscopic work. When using a monocular the unemployed eye should be kept open with accommodation relaxed, since squinting causes fatigue. A strip of cardboard 4 or 5 inches long, with an opening to fit over the tube of the microscope, leaving the other end to block the vision of the unused eye, will prevent the strain.

Illumination. Proper illumination is very important in microscopic work; unless the light is utilized to the best advantage the best results cannot be obtained. A north light, or, south of the equator, a southern light is desirable. Direct sunlight or an excessively bright light is to be avoided if possible or reduced by white shades or curtains. Artificial light is more practical because the intensity remains constant. A ground glass or blue filter should be used between the light source and the mirror, depending upon the type of light desired. A binocular microscope requires more light than a monocular; good results are unobtainable without sufficient light. However, too much light is as bad as not enough.

Correct Use of the Mirror. Use the flat side of the mirror for almost all purposes if there is a condenser on the microscope. In cases where the condenser is lacking, or a contour image is desired, as, for example, in hanging-drop preparations and fresh smears of intestinal protozoa or blood, the concave mirror is preferable. The mirror must be centered so that it is filled by the source of light and the maximum intensity of illumination is secured. The quality of the image is reduced if the mirror is used in any other position.

Correct Use of the Condenser. The condenser should be kept at almost its highest elevation. Only when contour images are desired should it be lowered. With high powers the iris diaphragm is used to cut down the light, never the condenser. The sharpest image is obtained when the aperture is the smallest, but never close the iris diaphragm to a point where the cone of light it delivers has a lesser optical angle than that of the objective used or the value of N.A. will be lowered.

Focusing. When using the high dry or oil-immersion objectives it is very important to focus on the preparation in the following way. Lower the objective with the coarse adjustment until it is almost in contact with the coverglass or slide, controlling it with the eye on a level with the stage. Then, looking through the eyepiece, elevate the objective until the object comes into view, and focus sharply with the fine adjustment. Never use the fine adjustment to lower the objective. Contact cannot be felt and the coverglass is apt to break. This ruins the preparation and may injure the lens. Particular care is necessary in focusing hanging-drop preparations since the coverglass is easily broken and there is the risk of infection if virulent organisms are present. Very few microscopes are parfocal. Use caution in shifting from one objective to another.

Care of the Microscope. The following precautions should be observed in order to prevent injury to the microscope:

1. If the fine adjustment works through the arm of the microscope, always grasp the instrument by the pillar which supports the stage. In those microscopes, however, which are not constructed in this way the arm has a handle portion made to serve in lifting the instrument.

2. Always keep the microscope in its case or covered with a bell jar or cloth when not in use in order to keep away the dust.

3. Care should be observed to keep all parts of the microscope from coming in contact with acids, alkalies, chloroform, xylol, and alcohol.

4. See that the lenses are clean before using the microscope. This applies especially to the upper and lower lens of the ocular and the lower lens of the objectives. Always use lens paper in wiping off the dust from dry objectives and the oil from immersion ones. Remove immersion oil before putting away the microscope. Dried oil becomes gummy and is hard to remove without danger to the lens. It can be removed by wiping with lens paper moistened with a drop of xylol or chloroform, but the cleaning should be done as rapidly as possible, with a final wiping off with dry lens paper, to avoid damage to the setting of the lenses. Throw lens paper away after using it once.

5. Lenses are very apt to deteriorate in the Tropics. One should be careful to protect the instrument from the direct light of the tropical sun.

6. If any oil is used on the mechanical parts for lubrication, all excess should be wiped off to avoid the catching of dust or gritty particles. Never use immersion oil to lubricate a microscope.

7. Always be certain that the slide is placed right side up on the microscope. The high powers cannot be brought into focus and there is danger that a lens may be damaged when the nosepiece is rotated on a slide which is upside down.

Darkfield Illumination

This method of lighting is used primarily for the examination of objects either too transparent or too small to be seen by light which passes through them. It is of great value in studying spirochetes, trypanosomes, etc. The objects in the field are illuminated only by light which enters obliquely and are seen by the rays which are reflected to the eye; they appear as brilliant silvery objects against a dark background. Particles beyond the range of resolution by direct illumination (less than 0.2μ) may be made visible as points of light. These are, however, diffraction images and not the actual objects.

Illumination. The stronger the light the smaller the objects that can be seen with a darkfield. A brilliant source of illumination is essential. A small arc lamp is the most satisfactory but a good gas light, a strong flashlight, or direct sunlight can be used. The rays should be parallel when they enter the condenser. Special darkfield equipment has the light source incorporated in the apparatus below the condenser. These lights are compact and convenient, and are adequate for all but the finest work, in which the more intense arc light gives better results. In using direct sunlight, it is necessary to place the mirror where it will receive the full sunlight while the rest of the microscope is shaded. If filters are used they must be transparent, never semi-opaque or ground glass.

Condenser. Darkfield illumination is usually secured by the use of a special condenser, the center of which is covered by an opaque area so adjusted that no direct rays from the light source can enter the eye. A small round piece of black paper, just large enough to block out direct light, placed in the center of the glass disc that fits into the ring under the lens of the ordinary condenser will work with the lower-power objectives but not with the higher ones. The special darkfield condenser, when used to replace the ordinary one, must be so adjusted that it can be elevated high enough to make contact with the bottom of a slide on the stage. The light is then adjusted and the condenser centered usually by means of set screws operating against springs, and a small circle etched in the surface of the upper lens. This circle is focused upon by the low-power objective (10× ocular) and appears as a bright luminous ring in a dark field. *Caution:* Do not be misled by the series of concentric rings in the condenser which focus at a lower plane than the single bright centering ring. The bright ring should be centered in the middle of the field by means of the centering device. *Note:* The luminous ring

cannot be seen unless the top lens of the condenser is faultlessly clean. Every trace of oil or dirt must be removed before use.

Most microscopes are equipped with a darkfield stop which may be inserted into the ring under the ordinary condenser. This serves admirably for use with either the low or high power objective. If special darkfield condensers are to be purchased, an authority should be consulted before a choice is made.

Objectives. The objectives usually used for darkfield examinations are the oil-immersion and the high dry. With condensers designed for use with a N.A. of 1.00 or below (almost all) the oil-immersion objective must be reduced in N.A. to below 1.00 by use of a "funnel-stop" inserted into the back mount of the objective, or by means of an iris diaphragm if the objective is so equipped.

Slide and Coverslip. Darkfield condensers are constructed to work with slides of a definite thickness, usually somewhere between 1.10 and 1.45 mm. Unless the correct thickness is used the cone of light cannot be properly focused upon the object and the results are unsatisfactory. The slide and coverslip must be perfectly clean and free from scratches. A No. 1 or even 0 coverglass should be used.

Preparation of Materials for Examination. The layer of fluid to be examined must be thin, and it must not contain too many objects or the whole background will be brightened and the objects indistinct. Air bubbles must be avoided.

Use of the Darkfield. After making all the preparations described above proceed as follows:

1. Place a good drop of immersion oil on top of the condenser and lower it a little.
2. Place slide on stage and raise condenser until there is a good contact between the bottom of the slide and the top lens of the condenser. This contact must be free from air bubbles.
3. Place drop of oil on coverglass and lower oil-immersion lens into it. This contact must also be free from air bubbles. Focus carefully. *Note:* In routine laboratory procedures it is seldom necessary to use oil-immersion objectives—high dry and low powers proving entirely adequate. It is recommended that the low power objective be employed in procedures such as observing the results of agglutination tests in leptospirosis. Many individuals experienced in darkfield microscopy feel that the method is of great value in studying many types of organisms not mentioned above.
4. If the light does not seem perfect, move the condenser up or down a little and attempt to find a spot where the light is better.

Micrometer

Micrometry, or the determination of the dimensions of an object microscopically, is often very useful in hematology, bacteriology, and particularly in animal parasitology. The unit in micrometry is the *micron*. This is the $\frac{1}{1000}$ part of a millimeter, and is usually written μ. A millimicron is the $\frac{1}{1000}$ part of a micron, or 0.000001 millimeter, and is written $m\mu$.

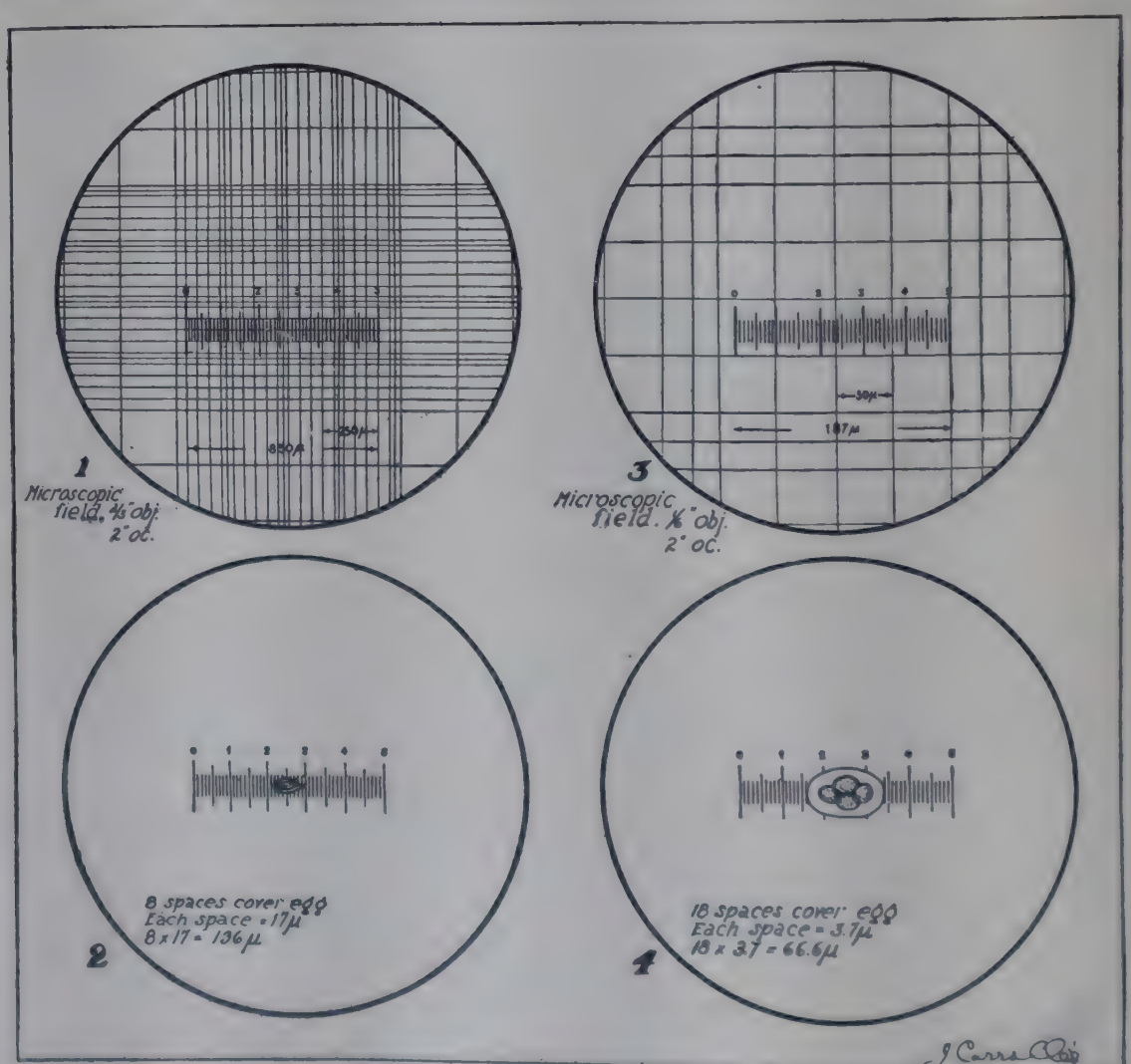
Ocular Micrometer. The most practical way of making these measurements is by placing a scale in the eyepiece. A number of types exist, the most common being the *disc micrometer* for insertion into the regular ocular, the *micrometer ocular* used to replace the normal eyepiece, or the *filar micrometer*. The disc micrometer is the least expensive and most commonly used type. The filar micrometer is the most accurate, but is expensive. The disc micrometer is a glass disc with ruled lines which is placed on the diaphragm of the ordinary ocular *ruled surface down*. The ruling and specimen should be seen in perfect focus at the same time. If the graduations cannot be clearly seen the micrometer is probably inserted with the ruling face up or the diaphragm of the ocular is in the wrong position for the eyes. To determine if the latter is the case, unscrew the top lens of the ocular slowly. If the scale comes into focus, the diaphragm should organic

ered. If not, it should be raised. The ocular micrometer is usually ruled with 50 or 100 lines or spaces, separated by longer lines into groups of 5 and 10.

Calibration. All micrometers have an arbitrary length and the apparent length depends on the magnification. Consequently each one must be calibrated for use with *each combination of objective and eyepiece*. A stage micrometer or hemacytometer is used for this standardization. A stage micrometer has ruled lines separated from each other by $\frac{1}{10}$ mm. (100μ). Some of these $\frac{1}{10}$-mm. spaces are again ruled with 10 lines giving spaces which are only $\frac{1}{100}$ mm. (10μ) apart. If one does not have such a scale, however, a hemacytometer makes a very satisfactory substitute. In any system of ruling of a hemacytometer, whether it be Thoma-Zeiss, Türck, or Neubauer type, the smallest squares in the central ruling are used for counting red cells. These are in groups of 16, and each one is $\frac{1}{20}$ mm. or 50μ square. It is these 50μ spaces which are used in calibration.

Procedure for Calibration with a Hemacytometer. The principle is exactly the same as with a stage micrometer.

1. Focus on area of the hemacytometer with the smallest squares (50μ), noting that there are triple or double lines and single lines.
2. Place a single line of the hemacytometer so that it corresponds exactly with the zero line of the ocular micrometer.
3. Being careful not to move the hemacytometer or ocular look to the apparent right



(*1*) Fifty lines of ocular micrometer covering 17 small square spaces (50μ each) of hemacytometer. Each ocular micrometer space = 17μ. (*2*) Schistosome egg fills 8 spaces of ocular micrometer. Egg is 136μ long. (*3*) Fifty lines of ocular micrometer cover 3.75 small square spaces. Each space = 3.7μ. (*4*) Hookworm egg fills 18 ocular micrometer spaces. Egg is 66.6μ long.

and note a second place where a line of the hemacytometer exactly coincides with one of the micrometer.

4. Note the number of lines on the micrometer (*a*) and the number of spaces on the hemacytometer (*b*) which thus are equal in distance.

5. Since each space on the hemacytometer is equal to 50μ multiply *b* by 50 and divide by *a*. This gives the value of each individual space on the micrometer *with the ocular, objective, and tube length employed in the calibration*. It must be recalibrated for every eyepiece-objective combination. *Note:* With the higher powers often there is no line to the apparent right on the micrometer which exactly coincides with one of the hemacytometer. In this case interpolation is required.

Example: If 20 lines of the ocular micrometer cover 4 spaces of the hemacytometer, then $4 \times 50\mu \div 20 = 10\mu$ = length of one space of the micrometer.

To measure the egg of an intestinal parasite, for example, simply focus on the egg and note the number of spaces covering it. Multiply this number by the value in microns of the space for the objective-eyepiece combination used.

Filters

There are several types of filters in common use for removal of bacteria from liquids.

Berkefeld Filters. These are made of diatomaceous earth, pressed into the shape of a hollow candle and cemented into a metal base which is drawn out into a tube. This is inserted in a glass mantle which holds the fluid to be filtered, and is attached by means of a rubber stopper to an ordinary suction flask or to some special device. There are three grades of porosity: V (viel), coarse (pores 8 to 12μ), is for clearing solutions and does not retain all bacteria. N, normal (pores 5 to 7μ), retains ordinary bacteria. W (wenig), fine (pores 3 to 4μ), retains bacteria and some viruses.

Mandler Filters. These filters, which are much used in the United States, are similar in construction and use to the Berkefeld filters. They are made of diatomaceous earth, asbestos, and plaster of Paris. There are three grades: "preliminary," "regular," and "fine," corresponding approximately to V, N, and W Berkefelds.

Chamberland Filters. These are made of unglazed porcelain (kaolin with a little sand). They are pressed into candles open at one end, into which the stem of a funnel can be fitted by means of a rubber stopper. The fluid filters through the candle from within outwards. There are nine grades: L 1 (coarse, like Berkefeld V, not retaining bacteria), L 1 bis, L 2, L 3 (like Berkefeld N, pores 2.7μ, retaining most bacteria), L 5, L 7, L 9, L 11, and L 13 (finest, retaining some viruses).

All of these filters are obtainable in various sizes.

Seitz Filters. These consist of a special asbestos pad which fits in a metal holder. The grade EK ("germicidal") removes ordinary bacteria. The discs are discarded when exhausted. These pads liberate alkali in the solutions, particularly the older types.

Ultrafiltration through specially prepared collodion membranes is much used in the study of viruses. The technic is complicated. (See Elford: *J. Path. & Bact.*, 34:505, 1931.)

Testing Filters. Gross defects (cracks, leaking joints) may be detected by immersing the candle in water with the closed end up and attempting to blow air through it. If it seems intact, assemble the filter, sterilize in the autoclave, and filter a liquid to which has been added sufficient of a 24-hour broth culture of prodigiosus (*S. marcescens*) or some similar organism to give about 100,000,000 organisms per ml. Culture liberal amounts of the filtrate. If no growth appears within 48 hours, the filter may be regarded as intact.

Cleaning Candles (Rivers' Method). If infectious material has been filtered, soak in some disinfectant such as cresol which does not coagulate protein. Scrub the surface with a brush, and force through the filter from within outward water (or salt solution if the fluid filtered contained globulin) until clean. Boil half an hour in 2 per cent sodium carbonate, and then in several changes of distilled water. Force water through the candle until it is clean and all alkali has been removed. If clogged with organic

material, Chamberland candles may be dried in a warm oven and gradually heated in a muffle furnace to a dull red heat, and then slowly cooled. Berkefeld filters often crack if so heated.

Filtration. This is better carried out by suction than by pressure. Filtration should be rapid, but the negative pressure should not exceed 35 to 50 cm. Hg. The liquid to be filtered should be cleared of detritus by preliminary centrifugation and filtration through paper, cotton, or a coarse filter.

The filtrability of a particle depends only in part upon the relative size of the particle and the pores of the filter. The composition and reaction of the liquid medium is equally important (see p. 189). If the medium is acid, the filters will usually retain small bacteria and some viruses which will pass through if it is slightly alkaline. Filtration of viruses is facilitated by suspending them in meat infusion broth or in 10 per cent serum rather than in salt solution, or by first drawing some sterile broth through the filter.

Capillary Pipets

With the possible exception of the platinum loop, there is no piece of apparatus so generally useful as the capillary pipet. It is made from a piece of ¼-inch soft-glass tubing, about 6 inches long. Held by the ends and constantly revolved, this is heated in a Bunsen flame, preferably fitted with a fish-tail tip, until it becomes soft in the center. It is then removed from the flame and, with steady traction, drawn out so that there intervenes a capillary portion 18 to 20 inches long. When cool, it is filed and this capillary portion broken off in the middle. One then has two capillary pipets. By using a rubber bulb, such as comes on medicine droppers, one has a means of sucking up and forcing out fluids. The bulb should be pushed on about ½ to ¾ inch; this gives a firmer surface to control the pressure on the bulb.

Bacteriologic Pipet. A bacteriologic pipet is made from a piece of tubing 9 inches long. The tubing is heated successively in the flame points 3 inches from each end; in each instance the tube is drawn out just sufficiently to make a constriction. Then, following the procedure described in the preceding paragraph and heating the middle of the tube, two capillary pipets are obtained. A piece of cotton is lightly pushed into the larger end.

Wright's Tube. This tube, with a hooked end which permits hanging the crook on the centrifuge guard, is the best known apparatus for securing small quantities of blood for serum tests. By filing and breaking the thicker part of the tube, the serum is made directly accessible to a capillary rubber-bulb pipet, or to the tip of a hemacytometer pipet, thereby facilitating dilution of the serum.

Lyon's Blood Tube. To make this tube, a 5- or 6-inch section of ½-inch tubing is heated in the center and drawn out as for making two bacteriologic pipets. This is divided, and the large end sealed off in the flame. Next the capillary end is sealed off. Then a very small flame is applied to a point on the large end just before it begins to taper to the capillary part. The heat causes the air sealed off inside to force out a blow hole. To use, the sealed capillary end is broken off and allowed to suck up blood from a drop just as with the Wright tube. I consider this tube superior to that of Wright.

Although these pipets may be sterilized during the flaming, and used immediately afterward, it is better to keep on hand a supply, suitably wrapped and autoclaved for use on occasion.

Apparatus and Technics in Anaerobic Methods

The following methods and equipment are best adapted to small diagnostic laboratories.

When organisms can be grown in liquid matrix and surface colonies are not necessary, use deep tubes of Brewer's medium, semisolid agar, or cooked meat. Anaerobiosis can be assured for the last two if the tubes are heated to drive off oxygen prior to inoculation and sealed with paraffin oil during incubation. By making the semisolid agar fairly stiff

it is possible to obtain isolated colonies in the depth of the medium. These can be reached by warming the tube gently and sliding the entire mass out into a Petri plate. Then with a sterile scalpel the mass can be sectioned at the desired level.

If it is desirable to obtain surface colonies on solid media it is best to use a system containing pyrogallic acid. Take two evaporating dishes at least 1 inch deep and have one at least an inch greater in diameter than the other. Pour the desired medium into the smaller of these exactly as is done with a Petri dish. After inoculating the surface, place about 1 Gm. of pyrogallic acid in the large dish and invert the smaller over it. Now run in a few milliliters of 5 per cent sodium hydroxide and immediately seal the space between the two dishes with paraffin oil. The same technic can be used for slants by cutting the plugs off flush with the tube, pushing them down, adding pyrogallic acid and hydroxide solution, and putting in a tight stopper. Incubate in an inverted position.

When it is necessary to provide an anaerobic atmosphere for a large number of cultures use a jar or can which can be tightly sealed. In the bottom of this place 1.5 Gm. powdered chromium and 0.5 Gm. sodium carbonate, per liter capacity of the jar; use a small beaker to contain the reagents. Into this powder pipet 15 ml. of 15 per cent (by volume) sulfuric acid. Place the cover on loosely until effervescence is about completed and then tighten it down and seal with Scotch tape or paraffin. Hydrogen gas is given off during the reaction and remains during the period of anaerobiosis; consequently, keep the container away from flames. An indicator which will demonstrate loss of anaerobic condition can be made by adding 2 drops of Loeffler's alkaline methylene blue to a tube of glucose broth and heating until the oxygen is driven out. Return of the blue color indicates the presence of oxygen.

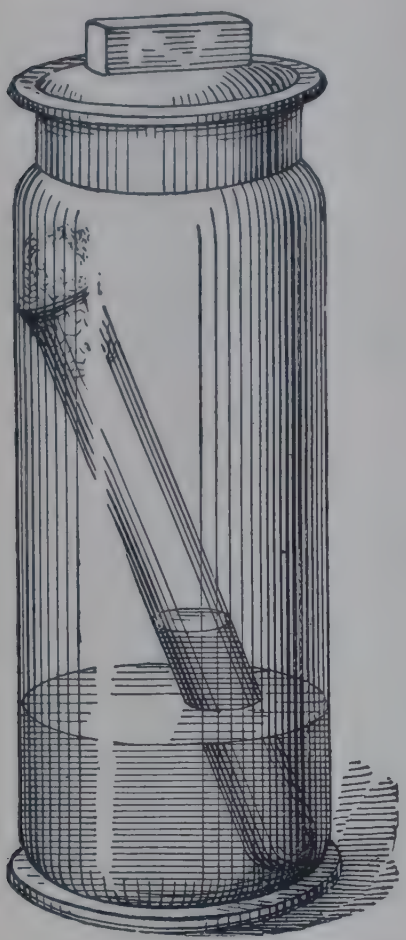
Novy Jar. This is one of the most convenient and satisfactory devices for securing strict anaerobic conditions. Any one of several methods described may be used for abstraction of oxygen. A good substitute may be improvised from an ordinary museum jar, the top of which is perforated to admit a rubber stopper carrying one or two glass tubes through which air can be withdrawn and hydrogen introduced. (A glass stopper is better.)

Method of McIntosh and Fildes. This method or one of its modifications is now regarded as giving the most satisfactory anaerobic conditions obtainable. It depends upon the oxidation of hydrogen by the oxygen in the jar due to the catalytic action of platinized, or preferably palladinized, asbestos wool. The culture tubes or plates are put in the jar. The asbestos wool, protected by a small cage of copper gauze, is heated red hot and suspended in the jar from the lid. The jar is quickly closed tightly, and sealed with plasticine or some substitute. A stream of hydrogen from a cylinder provided with a good reducing valve is then introduced so that it passes over the asbestos wool *very slowly* to avoid an explosion. This is continued until the oxygen is exhausted, and the inlet tubes are then closed. As an indication that an adequate degree of anaerobiosis has been secured McIntosh and Fildes suggested using a tube of sterile 2 per cent dextrose alkaline broth tinged with methylene blue. This is decolorized (reduced) when the oxygen is abstracted, and regains the blue color when oxygen is admitted.

Simple *replacement of air by hydrogen* from a cylinder or from a Kipp generator serves fairly well if palladinized asbestos is not available. It is more effective alternately to exhaust the air from the jar with an ordinary suction pump, and replace with hydrogen several times.

Buchner's Method. In this method 1 Gm. each of pyrogallic acid and caustic potash or soda for every 100 ml. of space in the vessel containing the culture is used to absorb the oxygen. It is convenient to drop in the pyrogallic acid; then to put in place the inoculated tubes or plates; then quickly to pour in the amount of caustic soda, in a 10 per cent aqueous solution, and immediately to close the containing vessel. A large test tube in which a smaller one containing the inoculated medium is placed, and which may be

closed by a rubber stopper, is very convenient. A good rubber-band fruit jar is satisfactory. A desiccator may be used for plates.



Arrangement of tubes for cultivation of anaerobes by Buchner's method. (MacNeal.)

Tarozzi's Method. In this method, pieces of fresh sterile organs are added to broth. Pieces of kidney, liver, or spleen are best suited. After adding the tissue the media may be heated to 80° C. for a few minutes without interfering with the anaerobic-producing properties of the fresh tissues. This is also a feature of Noguchi's method of culturing *Treponema pallidum*.

To get effective anaerobiosis it is necessary to put the pieces of tissue in the bottom of deep tubes of plain or glucose agar or broth, and to pour over the top sterile paraffin oil, or preferably melted petroleum jelly or paraffin. Liquid oil retards but does not prevent diffusion of oxygen into the medium. Such tubes can be put in a Novy jar and the oxygen largely exhausted by replacement with hydrogen or by the pyrogallic acid method.

Increased Carbon Dioxide Tension. Many organisms appear to grow better when the surrounding atmosphere contains more carbon dioxide and water vapor than is ordinarily present. The candle jar satisfies these conditions most easily. Plates and flasks are placed in wide-mouthed jars or cans equipped with tight-fitting tops. A small piece of candle is placed in with them and lighted. The top is secured tightly while the candle is still burning. When candles are not available a pledget of cotton soaked in alcohol will serve the purpose. Single culture tubes can be provided with increased carbon dioxide by cutting the plugs off flush with the tube and pushing them down into the lumen. A few drops of alcohol are then placed on the plug, ignited, and a rubber stopper inserted.

Partial Oxygen Tension. By incubating deep tubes of glucose agar which have been melted and cooled just before inoculation, without sealing with oil or paraffin, gradations of partial oxygen tension are obtained from aerobic conditions at the surface to practically complete anaerobiosis at the bottom. Some strains of streptococci, for example, grow only in a restricted zone below the surface.

B. Preparation of Tissues for Examination in Microscopic Sections

The most important step in the preparation of sections of tissues for histologic examination is proper and immediate fixation. This step in the technic is often in the hands of the surgeon at the time of the operation or the physician at autopsy, and it should be understood by them that a satisfactory diagnosis can be made only when the pieces of tissue are *at once dropped into a fixative*. Various protozoa, as amebae, disintegrate in one or two hours unless properly fixed, and body cells show degeneration after the tissues have been left without fixation for a few hours, which changes may be interpreted as pathologic.

Drop into the solution slices of tissue, not more than ¼ inch thick, *as soon as cut*. Leave in the fixative for 24 hours or longer when the specimen is to be sent away to a laboratory for diagnosis. *The pathologist will attend to the other steps.*

We use two fixation solutions in routine work, one of 10 per cent formalin and one of Zenker's solution. This latter requires prolonged washing of tissues following fixation and has little advantage over formalin for ordinary purposes.

Fixation. The piece of tissue to be fixed must not be too large. Using a sharp scalpel,

or preferably a razor, a section of tissue about ½ inch square and not more than ⅛ inch thick should be dropped into the bottle containing the fixative. The bottom of this bottle should have a thin layer of cotton with a piece of filter paper covering it. There should be at least twenty times as great a volume of fixing fluid as of tissue to be fixed. Delicate tissues, as pieces of gut, should be attached to pieces of glass, wood, cardboard, or blotting paper before being placed in the fixative. In fixing certain specimens of tissue, especially pieces of slit intestine, it is a good plan to lay the specimen, while wet, peritoneal side downward, on a piece of thick dry filter paper or lintless blotting paper; this prevents the specimen from being curled up. The whole is put into the fixing fluid, and the paper removed after fixation. The number or name of the specimen may also be written on the paper.

Formalin. The most convenient fixative for routine purposes is a 10 per cent solution of ordinary commercial formalin (4 per cent of formic aldehyde gas), either in water or, preferably, in 0.85 per cent salt solution. Fixation is complete in from 12 to 24 hours. By placing in the incubator at 37° C., 2 to 12 hours in the formalin solution suffices. If fixed in the paraffin oven (56° C.), fixation is accomplished in about one-half hour. Formalin once used for fixation must be thrown away.

Zenker's Fluid. Zenker's fluid probably gives the best histologic pictures and is the most satisfactory fixative for hematoxylin staining. It is a modification of Müller's fluid. The formula is:

Potassium bichromate	2.5 Gm.
Mercuric chloride	5.0 Gm.
Water	100.0 ml.

Just before use add glacial acetic acid 5 ml.

Zenker's fluid fixes in about 24 hours. After all corrosive sublimate fixatives one should wash the tissues in running water for 12 to 24 hours. The precipitate of mercury in the tissues is best removed by treating the section on the slide with Lugol's solution, rather than the tissue in bulk with iodine alcohol. A saturated corrosive-sublimate solution in salt solution with the additional 5 per cent glacial acetic acid may be used as a substitute for Zenker's fluid.

Alcohol. Where the tissue is to be examined chiefly for bacteria absolute alcohol is the best fixative. The piece of tissue should be small, not over ⅛ inch thick, and is to be suspended by a string to the cork so as not to lie on the bottom where the alcoholic strength tends to become weaker. Better histologic details are secured by fixing for two hours with 80 per cent alcohol and then transferring to absolute for 12 to 24 hours.

Note: Most laboratories prefer to receive tissue that has been fixed in formalin. Alcohol interferes with frozen sectioning and *postal regulations forbid alcohol in mail.*

Dehydration. After washing for 12 to 24 hours in running water following corrosive sublimate fixation, or simply washing for a few minutes after formalin, the tissues should be placed in 70 per cent alcohol in which they may be kept indefinitely.

C. Equivalent-normal Solutions

An equivalent-normal solution contains the hydrogen equivalent of a substance, expressed in grams, dissolved in sufficient distilled water to make 1 liter. The hydrogen equivalent is the number of grams that will unite with 1 Gm. of hydrogen or its equivalent. For an acid, the hydrogen equivalent would be the molecular weight divided by the number of *replaceable* hydrogen atoms that it contains. For a base, it would be the molecular weight divided by the number of hydroxyl (OH) groups.

To make a normal (indicated by N) solution, dissolve in distilled water the proper amount of the substance, and make up the volume to exactly 1000 ml. Thus sodium hydroxide has one hydrogen equivalent: Na = 23, O = 16, and H = 1, so one dissolves 40 Gm. sodium hydroxide in distilled water, and makes the volume up to exactly 1 liter.

Again, oxalic acid has the formula $(COOH)_2.2H_2O$, with a molecular weight of 126; containing two carboxyl groups, it has two hydrogen equivalents and it is necessary to divide by 2. So we dissolve 63 Gm. in water, and make up to 1 liter.

Preparation. If a chemical laboratory is not accessible, one may prepare such solutions with an error so slight as to be unimportant in clinical work in the following manner: Select perfect crystals of oxalic acid, such as can be obtained in a drug store, and weigh out, on the most accurate apothecary scales available, 6.3 Gm. of the most perfect crystals in the bottle. Put these preferably in a volumetric flask, and make up with distilled water to 1 liter. The use of a measuring cylinder is less accurate. With care, this method should give N/10 oxalic acid in which the error is less than 1 per cent.

Sodium hydroxide being very hygroscopic, it is impossible accurately to prepare a normal solution directly by weighing the solid. Having N/10 acid at hand, an N/10 NaOH solution may be prepared by weighing an excess of the substance, about 5 Gm. stick caustic soda, and dissolving it in about 1100 ml. distilled water. By means of a pipet, place 10 ml. in a beaker, and add 6 drops phenolphthalein solution (1 per cent in 95 per cent alcohol). Fill a buret with N/10 oxalic acid, and run it into the NaOH solution until the violet-pink color is just discharged. It is well to repeat the titration, and use an average. Reading the number of milliliters of N/10 acid used, we can calculate the strength of the NaOH solution. If 10.5 ml. of oxalic acid solution were required, it would show that the NaOH solution was stronger than N/10, as only 10 ml. would have been necessary if it had been of N/10 strength. It is, therefore, necessary to dilute in the proportions of 10 : 10.5. To do this, take exactly 1000 ml. of the too concentrated NaOH solution, add 50 ml. distilled water to it, mix thoroughly, and there is then 1050 ml. of N/10 NaOH. Calculation: $10 : 10.5 = 1000 : x$.

It is sometimes desirable to use *carbonate-free NaOH,* and this may easily be prepared by dissolving 100 Gm. C.P. NaOH in 100 ml. distilled water. The NaOH dissolves completely, but any Na_2CO_3 present is insoluble. Let it settle, or it may be centrifuged. Of the clear supernatant solution obtained after sedimentation, 55 ml. dissolved in CO_2-free water sufficient to make 1 liter will give approximately N/1 NaOH. Standardize in the usual way. Such a solution must be protected from the CO_2 of the air by storing in a paraffin-lined bottle, the two-holed rubber stopper of which bears guard tubes, and a glass tube for siphonage, the latter being lined and coated with paraffin. The guard tubes comprise one containing soda lime (or NaOH solution) and one with H_2O, and are arranged in series so that, when siphonage is established, the soda lime removes CO_2 from the incoming air, and then the H_2O prevents the carrying of any alkali into the bottle.

Water can be freed from CO_2 by vigorous boiling for 15 to 20 minutes (not longer), or by aeration for several hours, the air sucked through having been passed through NaOH solution (or soda lime tubes) and water. If the water is exposed to the air, CO_2 will again be absorbed, so, if stored, it should be protected by the method described for the NaOH solution above, although the paraffin lining of tube and bottle is not essential.

Since Acidum Hydrochloricum, U.S.P., is about two-thirds water (68.1 per cent), in order to make N/10 HCl, which would require 3.65 Gm. absolute acid per liter, it is necessary to take about three times this amount of U.S.P. acid. Take 12 ml., and add distilled water to make 1100 ml. Place 10 ml. in a beaker, add phenolphthalein solution, and titrate. If 11 ml. of N/10 NaOH were required, it would be necessary to add 100 ml. water to 1 liter of the diluted acid. Calculation: $10 : 11 = 1000 : x$. Other acid and alkali solutions can be made in the same way as are those described.

D. Determination of Hydrogen-ion Concentration

The significance of the hydrogen-ion concentration and the nature of buffer solutions have already been discussed in the section on Acid-base Equilibrium (p. 798). The

hydrogen-ion concentration, abbreviated as $[H^+]$, or C_{H+}, is a measure of the intensity of the acidity of a solution, as contrasted with the amount of acid present as determined by titration. It is commonly expressed as a fractional part of a normal solution, which contains 1 Gm. of H per liter. Thus the $[H^+]$ of a 0.01 N solution of a strong acid, since most of the H is dissociated, is nearly 0.01. Since the $[H^+]$ of the body fluids and secretions is low, it is convenient and customary to substitute for these small fractions the logarithm of the reciprocal of the fraction; i.e., the pH.

Since appreciable although minute amounts of H are dissociated even in strong alkaline solutions (the amount diminishing as the alkalinity increases), the reaction of such solutions may also be expressed in terms of their pH. The stronger the acid the lower the pH. Thus the pH of a tenth normal solution of a strong acid would be approximately 1.0, while that of a tenth normal solution of a strong alkali would be nearly 14.

To measure the pH directly requires electrometric determinations which are impracticable for routine purposes. Instead it is customary to estimate it colorimetrically, by adding a suitable indicator to the solution and comparing the color obtained with that yielded by the same indicator in buffered solutions of varying but known pH.

Indicators. The color change of indicators is dependent upon the $[H^+]$, or its complement, the $[OH^-]$ of the solution, and for each indicator there is a definite range of pH through which there is manifested a gradual change from the full alkaline to the full acid tint. Table 83 gives this range for several indicators, those italicized being the ones especially recommended for this work by Clark and Lubs.

Table 83

COLOR CHANGE AND pH RANGE OF INDICATORS

Indicator	*pH Range*	*Color Change*	
		Acid	*Alkaline*
Thymol blue	1.2– 2.8	Red	Yellow
Töpfer's reagent (dimethyl-amino-azobenzol)	2.9– 4.0	Red	Yellow
Bromphenol blue	3.0– 4.6	Yellow	Blue
Congo red	3.0– 5.0	Blue	Red
Methyl orange	3.1– 4.4	Red	Yellow
Bromcresol green	4.0– 5.8	Yellow	Blue
Methyl red	4.4– 6.0	Red	Yellow
Litmus (azolitmin)	4.5– 8.3	Red	Blue
Cochineal	4.8– 6.2	Yellow	Lilac
Bromcresol purple	5.2– 6.8	Yellow	Purple
Alizarin	5.5– 6.8	Yellow	Blue
Bromthymol blue	6.0– 7.6	Yellow	Blue
Neutral red	6.8– 8.0	Red	Orange
Phenol red (phenolsulfonphthalein)	6.8– 8.4	Yellow	Red
Cresol red	7.2– 8.8	Yellow	Red
Thymol blue	8.0– 9.6	Yellow	Blue
Cresol phthalein	8.2– 9.8	Colorless	Red
Phenolphthalein	8.3–10.0	Colorless	Red
Thymol phthalein	9.3–10.5	Colorless	Blue

Buffers. By mixing solutions of proper buffer substances in suitable proportions, mixtures of any desired pH values may be obtained. A suitable indicator is chosen from the list, and a small amount is added to each of the mixtures and to the unknown. The result is a series of graded standard tints with one of which the tint of the unknown is matched. A rough estimate of the pH of the unknown can be obtained by systematically

testing it with different indicators, since reference to the list will show the pH at which their full acid or alkaline color may be expected.

Table 84 gives the proportions in which buffer solutions must be mixed in order to produce desired pH values. The citric acid-phosphate series was proposed by McIlvaine, but we have slightly varied the proportions in order to secure colorimetric correspondence with the buffer mixtures of Sørensen and of Clark and Lubs. The boric acid, KCl-NaOH series is that of Clark and Lubs, and the resultant mixtures in it are to be diluted to 200 ml. before use. Use $Na_2HPO_4.2H_2O$ (Sørensen's phosphate) in M/5 strength; M/10 citric acid; a solution containing 12.4048 Gm. boric acid and 14.912 Gm. KCl per liter; and M/5 NaOH.

The chemicals employed in the preparation of such solutions must be specially purified—a task probably not within the ability of the usual clinical laboratory. The NaOH solution must be prepared and stored as indicated on p. 922. We will not give the details of this purification inasmuch as the laboratory with the equipment and experience necessary for this will have access to the literature. The buffer solutions and indicator solutions are readily purchasable, and, for the usual laboratory, we would advise that they be so obtained.

Sørensen's M/15 phosphate mixtures are also much used for pH range from 5.8 to 8.2.

Dissolve 9.08 Gm. of KH_2PO_4 in 1 liter of water.

Dissolve 9.47 Gm. anhydrous Na_2HPO_4 in 1 liter of water.

Prepare mixtures according to Table 85.

Procedure. Select test tubes of clear resistant glass, uniform in diameter, by introducing exactly 10 ml. water into each, and selecting those in which it rises to the same height. It is well to make permanent marks on these tubes at 5 ml. and 10 ml. Into a series of these tubes introduce 10 ml. (or 5 ml.) of standard buffer solutions selected from the range of the pH anticipated in the solution to be tested. In three additional tubes put an equal volume of this solution. It should be clear, and diluted up to five or ten times with distilled water if highly colored. To each standard tube and to one tube of the unknown add, with a pipet or medicine dropper held vertically, equal volumes of a suitable indicator solution and mix.

For the indicators that they recommend Clark and Lubs suggest 5 drops for 10 ml. of solution. The concentration of the indicator solution should be 0.02 per cent for cresol red, phenol red, methyl red, and cresol phthalein; for the others, 0.04 per cent. The solutions are prepared by grinding 0.1 Gm. of the dry dye in a mortar with N/10 NaOH, using the following volumes of alkali: For cresol red, 2.88 ml.; phenol red, 3.1; methyl red, 4.07; bromphenol blue, 1.64; bromcresol purple, 2.78; and thymol blue, 2.38. These are diluted with distilled water to 500 ml. to make a 0.02 per cent solution; to 250 ml. if a 0.04 per cent solution is desired.



Block comparator. (Courtesy, A. H. Thomas Co.)

Precise determinations require many precautions as regards background, source of light, exclusion of adventitious light, etc. It is very advantageous to use a wooden comparator block, as illustrated. We prefer to use diffuse daylight if possible and to back the comparator block with ground glass plate or with thin, plain unglazed white paper. In colorimetric comparisons the unknown and standard solutions must be balanced as regards any factors that would affect the shades of color compared. These are particularly intrinsic color or turbidity in the unknown solution and the thickness of the water columns. To avoid these errors, arrange the tubes in the block thus: In the two lateral spaces next to the observer put the tubes containing the unknown solution without indicator; in the middle space, a tube of water. In the spaces next to the light put, in the middle space the unknown solution containing indicator; in the lateral spaces standard tubes, changing the latter until the one is found which most nearly matches the color of

Table 84

PROPORTIONS OF BUFFER SOLUTIONS PRODUCING DESIRED pH

pH	*M/5 Phosphate, ml.*	*M/10 Citric Acid, ml.*	*pH*	*Boric acid-KCl, ml.*	*M/5 NaOH, ml.*
2.2	0.40	19.60	7.8	50.0	2.61
2.4	1.24	18.76	8.0	50.0	3.97
2.6	2.18	17.82	8.2	50.0	5.90
2.8	3.17	16.83	8.4	50.0	8.50
3.0	4.11	15.89	8.6	50.0	12.00
3.2	4.94	15.06	8.8	50.0	16.30
3.4	5.70	14.30	9.0	50.0	21.30
3.6	6.23	13.77	9.2	50.0	26.70
3.8	6.77	13.23	9.4	50.0	32.00
4.0	7.40	12.60	9.6	50.0	36.85
4.2	7.99	12.01	9.8	50.0	40.80
4.4	8.42	11.58	10.0	50.0	43.90
4.6	8.82	11.18			
4.8	9.50	10.50			
5.0	9.86	10.14			
5.2	10.52	9.48			
5.4	10.94	9.06			
5.6	11.37	8.63			
5.8	11.85	8.15			
6.0	12.43	7.57			
6.2	12.82	7.18			
6.4	13.22	6.78			
6.6	14.50	5.50			
6.8	15.40	4.60			
7.0	16.47	3.53			
7.2	17.12	2.88			
7.4	17.78	2.22			
7.6	18.45	1.55			
7.8	18.95	1.05			
8.0	19.15	0.85			

Dilute each mixture to 200 ml.

Table 85

SØRENSEN'S M/15 PHOSPHATE MIXTURES IN pH RANGE FROM 5.8 TO 8.1

pH 20° C.	*M/15 Na_2HPO_4 ml.*	*M/15 KH_2PO_4 ml.*	*pH 20° C.*	*M/15 Na_2HPO_4 ml.*	*M/15 KH_2PO_4 ml.*
5.8	8.0	92.0	7.0	61.1	38.9
5.9	9.0	91.0	7.1	66.6	33.4
6.0	12.2	87.8	7.2	72.0	28.0
6.1	15.3	84.7	7.3	76.8	23.2
6.2	18.6	81.4	7.4	80.8	19.2
6.3	22.4	77.6	7.5	84.1	15.9
6.4	26.7	73.3	7.6	87.0	13.0
6.5	31.8	68.2	7.7	89.4	10.6
6.6	37.5	62.5	7.8	91.5	8.5
6.7	43.5	56.5	7.9	93.2	6.8
6.8	49.6	50.4	8.0	94.7	5.3
6.9	55.4	44.6	8.1	95.8	4.2

the unknown. Interpolate if necessary. One must not rely on a color match with the end tube of a standard series. The result must be checked by using another series so selected that the pH of the unknown falls well within its range.

It is imperative that the glassware be chemically clean, that the chemicals used be the purest obtainable, and that the distilled water be neutral and free from CO_2. If the standard solutions are sealed in ampules of resistant glass having exactly the same diameter as the tubes used for the unknown solutions, and if they are kept in a dark cool place when not in use, they may usually be used for several months or more, but the colors should be checked occasionally.

Examination of Urine. The pH of the urine changes very rapidly on exposure to the air, because of the escape of CO_2. To get really accurate determinations it must be preserved (and if possible be collected) under oil, and examined under oil. In a tube put 8 ml. distilled water, add the indicator solution, and cover with oil. With a pipet introduce 2 ml. of urine, keeping the tip of the pipet under the surface of the water. Then proceed as above. The usual pH is about 6, the range being from about 4.8 to 7.5. Bromcresol green or purple may be used for acid specimens, bromphenol blue for average acidities, and phenol red for alkaline specimens.

Bicolor Method. The bicolor method of determining the pH has many advantages over the use of buffer solutions. The standard solutions are more permanent, are less affected by variations of temperature, and errors due to impurities in the buffers are eliminated, although pure indicator solutions are essential. The method has been elaborated by Gillespie and by Hastings et al. (1925) and applied to determinations of the pH of the urine and blood plasma, and it yields accurate results. (For details consult original article or Peters and Van Slyke: *Quantitative Clinical Chemistry.*)

Table 86

DETERMINATIONS OF pH VALUES WITH BICOLOR METHOD

Drop Ratio (Front: Back)	*pH Values with*			
	Methyl Red (0.008%)	*Bromcresol Purple (0.012%)*	*Phenol Red (0.004%)*	*Thymol Blue (0.008%)*
1 : 9	4.05	5.3	6.75	7.85
2 : 8	4.4	5.7	7.1	8.2
3 : 7	4.6	5.9	7.3	8.4
4 : 6	4.8	6.1	7.5	8.6
5 : 5	5.0	6.3	7.7	8.8
6 : 4	5.2	6.5	7.9	9.0
7 : 3	5.4	6.7	8.1	9.2
8 : 2	5.6	6.9	8.3	9.4
9 : 1	5.95	7.2	8.65	9.75

Barnett and Chapman introduced a relatively simple procedure which illustrates the principles of the method and is sufficiently accurate for most practical purposes.

Place 18 test tubes in rack in 2 rows of 9 each. Beginning at the left in the front row, place 1 drop of indicator in the first tube, 2 in the second, and increase by 1 drop in each succeeding tube as one passes to the right. Treat the rear row similarly, but begin at the right and pass to the left. To each tube in the front row, add 1 drop (2 to 3 drops for the thymol blue series) N/20 NaOH; to each tube in the rear row, add 1 drop N/20 HCl (use 1 drop 2 per cent KH_2PO_4 for thymol blue series instead of HCl). Fill all tubes to 5 ml. level with water. This standard series is viewed from the front in such a manner that the line of vision traverses two tubes—one in the front row and its part-

ner in the rear row—the total amount of indicator in each pair being 10 drops. The composite colors form a graded series, and the preceding table shows the pH values represented by each pair. The methyl red solution is prepared by grinding in acid-free alcohol until dissolved, and then diluting 3 volumes with 2 volumes of H_2O. The preparation of the other indicators is given above.

In a tube containing 10 drops of indicator solution put sufficient of the unknown solution to bring the volume to 5 ml. and mix. Put in a comparator block and back with two tubes of water. Also put a pair of standard tubes in the block and back with a tube containing the unknown solution without indicator. Change the pairs of standard tubes until a pair is found which matches the color of the unknown. Since this pr[o]cedure necessitates looking through three tubes in series, the openings for inspec[tion] the tubes must be drilled lengthwise through the comparator block.

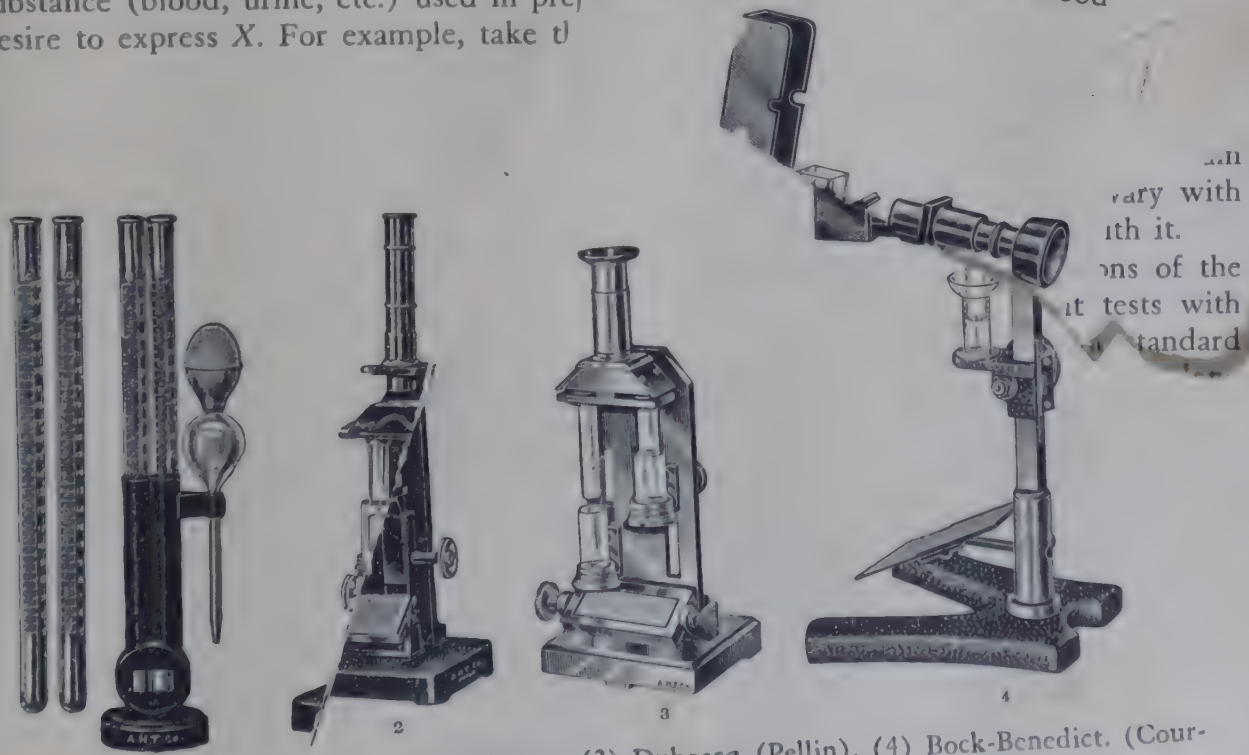
E. Colorimetric Determinations

There are two types of visual colorimeters in general use; in one, the [depth] of the solu- intensity of the colors of the two solutions is matched by varying the [ ] depth of the tions; in the other this is accomplished by diluting one of the solut[ions] fluid traversed by the line of vision remaining constant. [ ]ill vary inversely [ ]ula:

With the plunger type the relative concentrations of the [ ] as the readings (depths), and the calculation follows the s[ame]

$$\frac{S}{U} \times F = X$$

[ ]ndard and unknown solutions [ ]sult sought. F is constant under [ ] determined by three considera- in which S and U are, respectively, the readings [ ]of substance (nitrogen, uric acid, when colors are matched, F is a factor, and [ ] (2) the relative volumes to which the conditions of each determination, and [ ]he relation of the *actual* amount of tions, viz.: (1) The actual amount (in ter[ms] [ ]unknown to the terms in which we sugar, etc.) used in preparing the standard [ ]n q[uantity] nonprotein nitrogen of blood unknown and standard have been diluted, [ ]ulation for substance (blood, urine, etc.) used in prep[aration] desire to express X. For example, take t[he]



Types of colorimeter: (1) Myers. (2) Duboscq. (3) Duboscq (Pellin). (4) Bock-Benedict. (Courtesy, A. H. Thomas Co.)

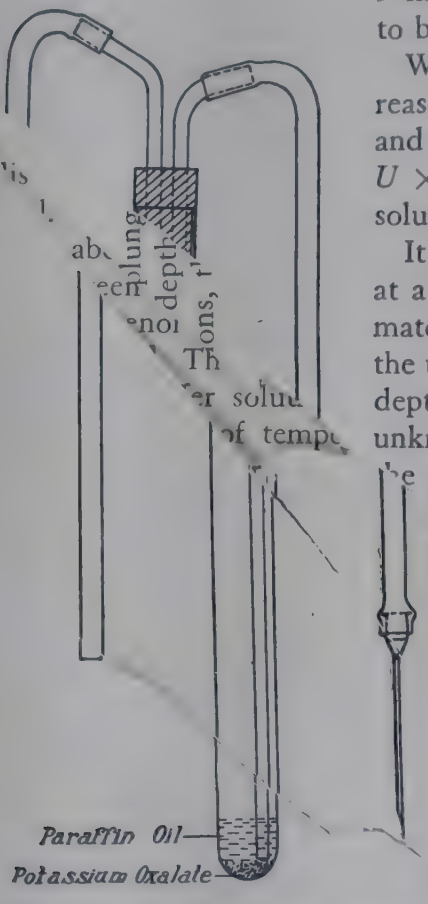
(see p. 766). S and U are determined, and we are to express X as milligrams per 100 ml. blood.

$$\frac{S}{U} \times 0.3 \times \frac{50}{100} \times \frac{100}{0.5} = \frac{S}{U} \times 30 = \text{milligrams nonprotein nitrogen per 100 ml. blood.}$$

(1) We have used 0.3 mg. nitrogen (not ammonium sulfate); (2) standard is diluted to 100 ml. and unknown to 50 ml.; therefore multiply by 50/100 (or divide by 2); (3) 5 ml. filtrate represents 0.5 ml. whole blood, and X is to be expressed in terms of 100 ml.

With the dilution type of colorimeter, the same reasoning applies, but we have here a direct proportion, and the general formula for the calculation becomes $U \times F = X$, if, as is usually the case, it is the unknown solution which is diluted.

It has been advised usually to set the standard solution at a fixed point and vary the depth of the unknown to match it. The calculation may be simplified by setting the unknown at some appropriate height and varying the depth of the standard. In the example above, e.g., if the unknown is set at 30, the reading of the standard gives the value of X directly.



Tube used in collecting blood. (*J. Biol. Chem.*, **30**:289, 1917.)

rmanent standards of tinted glass or of stable ns for practically all the examinations can be d, but very few are satisfactory. It is usually so to prepare the standard at the time of the pur the same reagents as for the unknown. pref nd unknown solutions should be handled test, way and finished practically simultaneously. Stand olorimeter take precautions against retinal in the dventitious light. Adjust the scales if In usin t they read zero when the cups are fatigue they just touch the plungers, or deter- factor. Make sure that the fields are

5.3	m
5.7	by
5.9	be
6.1	

ndard against itself. If the field is and the halves of the field made equally illuminated and check divided by a line, this line must equal in area.

If a regular colorimete is not availab ate results provided their in- test tubes can be use d quite satisfacto f fluid being measured by a ternal diameter is identical, the length or even graduated cylinders or ruler.

ctric Colorimeter. The principle of this type of instrument is Photoele epends upon the fact that when a is exposed to light a feeble d electric current is generated which is propor nsity of the light and can be measured with a sensitive galvanometer. ing a colored solution is interposed between the cell and the light the in ht reaching the cell is re- duced, and there is a corresponding reductio h of the current. The denser the color, the less the light reaching the c the current is reduced. It is thus possible to compare the color of an ution with that of a standard solution containing a known amount o to be measured. The comparative density of the colors is expressed by not a simple arith- metic ratio between the strengths of the currents.

Owing to the technical difficulties and expense inv ng a galvanometer sufficiently sensitive and accurate for this purpose, m ments now in use

are provided with two identical photoelectric cells so arranged in series with one another (and with a galvanometer and a calibrated variable resistance) that when the cells are equally illuminated and the instrument is properly adjusted, the currents produced by the two cells balance each other and there is no flow through the galvanometer. If a tube containing a colored solution is interposed between the light and one of the cells the current produced by this cell is reduced, the balance is upset, and a current will flow through the galvanometer. By adjusting the variable resistance the balance is restored and the galvanometer reading is brought to zero. The instrument is provided with a scale indicating the resistance in the circuit, and if the calibrations on the scale are spaced logarithmically, as in the Klett-Summerson colorimeter, and proper precautions are observed, the density of the colors is directly proportional to the scale readings, and no calibrated curves or charting on special semi-logarithmic paper are necessary. We have found this instrument simple, convenient, and adequate for the usual routine laboratory procedures.

In order to secure the necessary sensitiveness and accuracy, it is essential to insert a suitable light filter of colored glass between the light and the photoelectric cells. As a rule this should be one which removes most of the light except that fraction which is specifically absorbed by the solution being measured. Thus, if a red solution is being examined, which absorbs blue light but lets red come through, a blue filter should ordinarily be used to absorb the light in the lower (red) end of the spectrum. This increases markedly the difference in the scale readings obtained with two similar red solutions of different density and increases the sensitiveness of the instrument correspondingly. A similar advantage may be obtained by using such filters with visual colorimeters, but this is not ordinarily done in routine work. The proper filter varies with the solution to be tested and must be ascertained, either from the directions for use issued with the instrument employed, or elsewhere. If this is not certain, a test should be made with a number of different dilutions of known strength, and each read with several different filters. That filter should be chosen which gives readings which are most nearly proportional to the known quantities of material present. For most ordinary determinations with the Klett-Summerson colorimeter, three filters suffice: a blue filter with a special range of about 400 to 465mμ; a green filter with a range of 500 to 570mμ; and a red filter with a range of 640 to 700mμ. For certain determinations a special filter with a different or more restricted range may be necessary. Readings high on the scale (above 400 to 500) are not accurate, and should be lowered either by using smaller quantities of the material to be measured or by a different filter. Certain precautions must be observed in adjusting and using the instrument, which vary with the type of instrument and should be ascertained from the directions issued with it.

It is possible to construct a calibration curve, using several different dilutions of the standard of known strength and charting the results obtained. Subsequent tests with unknown solutions can be read directly from such charts without using a standard solution for each test. This is dependable only if the technic is so standardized by long practice that it is precisely repeated in every essential detail in every test. In the average laboratory where routine tests are made this is usually impracticable. It is far safer to set up a standard solution with each test, as minor differences in technic will ordinarily affect the standard and unknown solutions proportionately. The same objection applies to the use of colored-glass standards or similar makeshifts. If such curves are used for special reasons, the technician must practice with standard solutions until essentially identical scale readings can regularly be reproduced. New charts must be prepared with each new batch of reagents or other apparently minor change in procedure.

The chief advantages of this type of colorimeter are that readings can be made much more quickly and that accurate readings can be made with solutions too pale to read with a visual colorimeter. Determinations can therefore be made with smaller amounts of material. The photoelectric colorimeter also obviates errors due to retinal fatigue

or difficulties some individuals have in matching colors closely. The other potential errors inherent in colorimetric procedures remain, and results obtained are not necessarily more precise or dependable than those obtained with a visual colorimeter. If the solutions are in the slightest degree turbid, opalescent, or "off color," the error will be as great or often greater than with a visual colorimeter.

F. Anatomic and Physiologic Normals

In examinations in the pathologic or chemical laboratory the following may be considered approximately as normal findings:

I. Anatomic Normals. Averages.

Adrenals. Length, 2.4 to 2.8 inches (6 to 7 cm.). Breadth, 1.2 to 1.4 inches (3 to 3.5 cm.). Weight, 0.17 to 0.21 ounce (5 to 6 Gm.) each. Left usually larger.

Aorta. Length, varies, 17 to 20 inches (42.5 to 50 cm.). Thickness of wall, 0.06 to 0.08 inch (1.5 to 2 mm.). Diameter, 0.75 to 1.25 inches (1.7 to 3 cm.). Weight, 1.2 to 1.6 ounces (35 to 45 Gm.).

Appendix. Length, quite variable, 3.5 to 4 inches (9 to 10 cm.). Diameter 0.25 inch (6 mm.). Weight, 0.25 to 0.5 ounce (7 to 14 Gm.).

Bladder. Capacity, 16 ounces (500 ml.) when normally distended. Thickness of wall, 0.1 inch (2.5 mm.). Weight, 1 to 2.1 ounces (30 to 60 Gm.).

Brain. Weight, female 44 to 45 ounces (1250 to 1275 Gm.); male 48 to 51 ounces (1365 to 1450 Gm.). Length, 6.5 inches (16.5 cm.). Transverse diameter, 5.5 inches (14 cm.). Vertical diameter, 5 inches (12.7 cm.). Dimensions in female are 0.4 inch (1 cm.) less.

Esophagus. Length, 10 to 12 inches (25 to 30 cm.). Diameter of lumen, [illegible] inches (3 cm.). Thickness of wall, 0.3 inch (8 mm.). Weight, 1.4 ounces (40 Gm[illegible] [illegible]ually

Fallopian Tubes. Length, 3 to 5 inches (7.6 to 12.6 cm.). The right us[illegible] t[illegible] nger. Diameter of lumen averages 0.1 inch (2.5 mm.). [illegible]ould 1.5 ounces (30 to 45 ml.).

Gall-bladder. Length, 3 to 4 inches (7.5 to 10 cm.). Diameter, 1 to [illegible]lly s[illegible] 2.5 to 3 cm.). Thickness of wall, 0.04 to 0.07 inch (1 to 2 mm.). Capacity, 1 to[illegible] aga[illegible]ces (30 to 45 ml.).

Heart. Weight, female 8.8 to 9.8 ounces (250 to 280 Gm.), male 9.5 to 12.7 ounces (270 to 360 Gm.). Length, 4.5 to 5.5 inches (11.5 to 14 cm.). Breadth, 3 to 4 inches (7.5 to 10 cm.). Thickness, 2 to 3.1 inches (5 to 8 cm.). Thickness, wall left ventricle, 0.35 to 0.47 inch (9 to 12 mm.), right ventricle, 0.1 to 0.12 inch (2.5 to 3 mm.). Circumference, mitral orifice, 4.1 to 4.3 inches (10.4 to 10.9 cm.). Circumference, tricuspid orifice, 4.7 to 5 inches (12 to 12.7 cm.). Circumference, aortic orifice, 3 to 3.2 inches (7.7 to 8 cm.). Circumference, pulmonary orifice, 3.4 to 3.6 inches (8.5 to 9 cm.).

Intestines. Small intestine, length, 22.5 ft. (6.75 meters); 2/5 jejunum and 3/5 ileum. Diameter from 1.85 inches (47 mm.) in duodenum to 1.06 inches (27 mm.) at the end of ileum. Large intestine, length, 70.9 to 76.8 inches (180 to 195 cm.). Duodenum, length, 10.2 to 11.2 inches (26 to 28.5 cm.).

Kidneys. Weight, left, 5.3 ounces (150 Gm.), right, 5 ounces (140 Gm.). Thickness of cortex, 0.4 inch (1 cm.). Length, 4.5 inches (11.5 cm.). Breadth, 2.50 inches (6.2 cm.). Thickness, 1.25 inches (3.2 cm.). The left longer and the right thicker.

Liver. Weight 50 to 60 ounces (1440 to 1680 Gm.). Greatest transverse diameter, 7.9 to 9.5 inches (20 to 24 cm.). Greatest anteroposterior diameter, 3.9 to 5.9 inches (10 to 15 cm.). Vertical diameter, 5 to 6 inches (12.7 to 15 cm.).

Lungs. Weight, combined, 36 to 45 ounces (1020 to 1290 Gm.). Weight, male, right lung, 24 ounces (680 Gm.), left lung, 21 ounces (600 Gm.). Weight, female, right lung, 17 ounces (480 Gm.), left lung, 14.8 ounces (420 Gm.). Length, 10 to 12 inches (26 to 30 cm.). Anteroposterior diameter at base, 7 to 8 inches (17.5 to 20 cm.). Transverse diameter at base 4 to 5 inches (10 to 12.7 cm.). The right lung is shorter, broader and thicker than the left. Dimensions in the female average 1 inch (2.5 cm.) less.

Mammary Gland. Weight in adult, 5.25 to 7 ounces (150 to 200 Gm.). Weight during lactation, 14 to 31.75 ounces (400 to 900 Gm.).

Ovaries. Weight (each), 0.12 to 0.25 ounce (4 to 8 Gm.). Length, 1.5 inches (3.8 cm.). Breadth, 0.75 inch (1.9 cm.). Thickness, 0.5 inch (1.2 cm.).

Pancreas. Weight, quite variable, 2.1 to 4.8 ounces (60 to 135 Gm.). Length varies, average 6 to 8 inches (15 to 20 cm.).

Parathyroids. Length, 0.2 to 0.25 inch (6 to 7 mm.).. Breadth, 0.15 to 0.17 inch (3 to 4 mm.). Thickness, 0.05 to 0.075 inch (1.5 to 2 mm.).

Pineal Gland. Length, 0.4 inch (1 cm.). Breadth, 0.2 inch (5 mm.). Thickness, 0.2 inch (5 mm.). Weight, 3 gr. (0.2 Gm.).

Pituitary Body. Length, 0.3 inch (8 mm.). Breadth, 0.5 inch (1.2 cm.). Weight, 5 to 10 gr. (0.3 to 0.6 Gm.).

Prostate. Weight, 0.8 ounce (22 Gm.). Length, 1.25 to 1.5 inches (3.1 to 3.8 cm.). Breadth, 1.5 to 1.75 inches (3.8 to 4.5 cm.). Thickness, 1 inch (2.5 cm.).

Salivary Glands. Parotid, weight, 0.8 to 1 ounce (25 to 30 Gm.). Sublingual, weight, 0.06 to 0.09 ounce (2 to 3 Gm.). Submaxillary, weight, 0.25 to 0.3 ounce (8 to 9 Gm.).

Seminal Vesicles. Length, 2 inches (5 cm.).

Spinal Cord. Length, 18 inches (45 cm.). Weight, 0.9 to 1 ounce (27 to 30 Gm.). Transverse diameter averages 0.5 inch (1.2 cm.). Anteroposterior diameter averages 0.4 inch (9 mm.).

Spleen. Weight, 5.5 to 6.9 ounces (155 to 195 Gm.). Length, 4 to 5 inches (10 to 12.5 cm.). Breadth, 3 inches (7.7 cm.). Thickness, 1 to 1.5 inches (2.5 to 3.7 cm.).

Stomach. Capacity, 1 to 2 quarts (1 to 2 liters). Thickness of wall, 0.25 inch (6 mm.). Weight, 4.5 to 6.2 ounces (125 to 175 Gm.).

Testes. Weight, 0.65 to 0.8 ounce (20 to 25 Gm.) each. Length, 1.5 inches (3.8 cm.). Breadth, 1 inch (2.5 cm.). Thickness, 0.8 inch (2 cm.).

Thoracic Duct. Length, 15 to 18 inches (37.5 to 45 cm.).

Thymus Gland. Weight at birth, 0.5 ounce (13.7 Gm.) and increases to 0.9 ounce (26.2 Gm.) at end of second year when it gradually decreases until gland disappears. Dimensions at birth, length, 2.4 inches (6 cm.), breadth, 1.5 inches (3.7 cm.) and thickness 0.25 inch (6 mm.).

Thyroid. Transverse diameter, 2.4 to 2.8 inches (6 to 7 cm.). Height, 1.2 inches (3 cm.). Weight, 1 to 1.4 ounces (30 to 40 Gm.).

Ureters. Length, 11.2 to 12 inches (28 to 30 cm.). Slightly longer on left side and longer in male. Diameter of lumen varies, averages 0.1 inch (2.5 mm.).

Urethra. Male. Length, 6.4 to 8.25 inches (16 to 20.6 cm.). Prostatic, 1 to 1.25 inches (2.5 to 3.1 cm.); membranous, 0.6 to 1 inch (1.5 to 2.5 cm.) and the anterior 4.75 to 6 inches (12 to 15 cm.). Female. Length, 1.5 inches (3.8 cm.). Diameter of lumen averages 0.25 to 0.4 inch (7 to 10 mm.).

Uterus. (Virginal) length, 2.8 inches (7 cm.). Breadth, 1.6 inches (4 cm.). Thickness, 1 inch (2.5 cm.). Weight, 1.4 to 1.8 ounces (40 to 50 Gm.). The dimensions of a multiparous uterus are each increased 1 cm. or more and the weight is increased 0.7 ounce (20 Gm.). Length of cavity in virgin, 2 inches (5 cm.), in multiparae, 2.25 inches (5.7 cm.).

Vagina. Length, 3 to 3.5 inches (7.6 to 8.9 cm.). The posterior wall is slightly longer than the anterior.

II. Physiologic Normals (Adult).

Blood: (Values are in mg. per 100 ml. whole blood unless otherwise noted.)

Specific gravity	1.041 to 1.067 (1.026 to 1.032 for serum)
Reaction (see p. 798)	pH 7.3 to 7.5
Total solids	19 to 23%
Hemoglobin	15.6% (by weight) (females, 14.2%)

Serum albumin	3.8 to 5.2%
Serum globulin	2.0 to 3.5%
Fibrin	0.2%
Total nitrogen	2.6 to 3.5% (plasma, 0.6 to 1.1%)
Nonprotein nitrogen	25 to 39 (plasma, 20 to 30)
Urea nitrogen	12 to 18
Amino-acid nitrogen	6 to 8 (plasma, 4 to 7)
Ammonia nitrogen	about 0.1
Uric acid (Folin-Wu method)	2 to 3 (extremes, 1 to 4)
"Creatinine"	1 to 2
Creatine	3 to 5 (plasma, 0 to 3.8)
Sugar (Folin-Wu method)	70 to 120 (60–100 true)
Chlorides (as NaCl)	450–500 (plasma, 580 to 620)
Fat (Bloor's fat method)	about 600
Cholesterol (Bloor's method)	140–210
Lecithin (Bloor's "lecithin")	30 (plasma, 22)
Acetone bodies	0 to 4
Bicarbonate (plasma)	53 to 75 vol. % CO_2
Oxygen capacity	20.9 vol. %
CO_2 tension (arterial)	about 40 mm. Hg
Calcium	5.3 to 6.8 (serum or plasma, 9.0 to 11.0)
Magnesium	2.3 to 4 (serum or plasma, 1.6 to 3.0)
Sodium	170 to 225 (serum or plasma, 310)
Potassium	153 to 240 (serum or plasma, 18–21)
Phosphorus, total (as H_3PO_4)	about 120 (plasma, 35 to 40)
Phosphates, inorganic (as P)	(serum) 3.2 to 4.3
Sulfates (as S)	0.5 to 1.0
Diastase (see p. 797)	8 to 64 units

CEREBROSPINAL FLUID (see p. 749): (Values are in mg. per 100 ml. unless otherwise noted.)

Specific gravity	1.001 to 1.008
Pressure	5–12 mm. Hg or 70–160 mm. H_2O
Serum albumin	about 6
Serum globulin	20–30
Nonprotein nitrogen	15–35
Urea nitrogen	10–15
Creatinine	1–2
Sugar	50–80
Chlorides	720–750

STOMACH CONTENTS (see normal acidity curve, p. 864):
One hour after Ewald test meal:

Reaction	pH 0.9–1.6
Quantity	40 to 50 ml.
Total acidity	40 to 80
Free hydrochloric acid	25 to 50
Pepsin	64 to 256 Mett units

Residuum (fasting):

Quantity	20 to 100 ml. (rarely 150)
Total acidity	10 to 50
Free acidity	0 to 30
Pepsin	3 (Mett)

Trypsin	7 (Spencer)
Bile	present in about 60% of cases
Gastric mucus	traces

DUODENAL CONTENTS (see pp. 867–868).

URINE (24-hour specimen; values in Gm. unless otherwise specified):

Quantity	25 ml. per kg. body weight
Specific gravity	1.015 to 1.025
Reaction	acid to litmus (pH 4.8 to 8.0, average about 6.0)
Total solids	55 to 70
Total nitrogen	10 to 16
Urea	10 to 40 (supplies about 85% of total N)
Uric acid	0.2 to 2
Creatinine	1 to 1.5
Ammonia	0.5 to 1.2 (see NH_3 quotient, p. 839)
Hippuric acid	0.6 to 1
Albumin (see p. 828)	
Chlorides (as NaCl)	10 to 15
Sulfur (as H_2SO_4)	2 to 2.5
Phosphorus (as P_2O_5)	1 to 5 (about 90% of phosphates are acid phosphates)
Calcium (as CaO)	0.1 to 0.6
Magnesium (as MgO)	0.2 to 0.6
Acetone bodies	about 10 mg.
Sugar (see p. 833)	

FECES:

Average daily output, moist feces (Hawk)	110 Gm.
24-hour amount on ordinary mixed diet	110 to 170 Gm. (25 to 45 Gm. solids)
24-hour amount on vegetable diet	to 350 Gm. (about 75 Gm. solids)

Fatty substances (averages expressed as per 1 Gm. dried feces):

Total fat	150–250 mg.
Total fatty acid	90–130 mg.
Total soap	50–100 mg.
Total neutral fat	10– 20 mg.

RESPIRATION:

Alveolar air:

Oxygen	14.5%
Carbon dioxide	about 5.5% (35 to 40 mm. CO_2 tension)
Nitrogen	80%

Air hunger in diabetes or chronic nephritis begins only when CO_2 tension has fallen to 20 or 25 mm.

G. Important Diseases and Injuries Due to Toxic Plants

Ackee (Akee) Poisoning (Vomiting Sickness of Jamaica). This disease is caused by eating the unripe fruit ("akee") of the tree *Blighia sapida*. This tree is common in the West Indies, although it is believed to have been imported from West Africa. The ripe, naturally opened fruit is harmless and is valued as a food. According to Scott (1939) poisoning is caused by eating fruit which is not ripe, which has been picked from a dead branch or injured tree, or which has lain upon the ground. Illness begins acutely about two hours after ingestion with abdominal pain and vomiting. After a remission of a few hours there is a recurrence of vomiting and in the severe cases

convulsions, coma, and usually death. The poison is in the arillus (outer coat) of the fruit and according to Jordan and Burrows (1937) in the seeds. Evans and Arnold (1938) obtained from unripe arilli a saponin which was hemolytic, and highly toxic for kittens and guinea pigs in which it produced hemorrhages and fatty degenerative changes, particularly in the liver and kidneys. Arilli from fully opened fruit were nontoxic for these animals and nonhemolytic. The toxic effect is cumulative when sublethal amounts are taken at daily intervals. In the West Indies many deaths have been attributed to akee poisoning, but it seems evident that similar symptoms may follow the ingestion of other irritating or poisonous foods.

Arrow Poisons. Many primitive people add poison to the physical injury produced by their arrows. Although animal secretions, particularly snake venoms, have been used, they are inferior in effect to the vegetable poisons. In some arrow poisons snake venom is combined with the plant extract, but as the venom is often treated in such a way as to destroy the toxins, the effect must be due largely or entirely to the vegetable poison. Material of bacterial nature is also used, particularly the tetanus bacillus, but most of the potent arrow poisons are vegetal.

Acocanthera. This genus of the Apocynaceae includes several species which furnish a powerful poison widely used by the African natives. A decoction is prepared from the wood or roots, evaporated over a fire to a syrupy consistency, and painted over the heads of arrows. The active principle is ouabain, a glucoside related to those in digitalis, which speedily causes death from cardiac failure after a brief period of rapid irregular pulse, dyspnea, and often convulsions.

Strophanthus. Several species of this genus, also of the Apocynaceae, have been used by the African natives to produce arrow poisons. At least two species, *S. sarmentosus* and *S. gratus,* are found in Liberia, growing as bushes with striking pink pentacle-like flowers. The seeds are cooked in water, the extract evaporated to a syrup, and often mixed with the heads of snakes and sometimes with a little vegetable resin. The active principle, strophanthin, is a glucoside closely related to ouabain, and causes death from injury to the heart muscle. Both drugs have been used in small doses as cardiac stimulants as a substitute for digitalis.

Strychnos. The principal arrow poison of the Amazon river tribes, curare, is obtained by extracting the bark of various species of *Strychnos.* According to A. Hamilton Rice, the sheath or rind of a vine, called Itary-cipo (*S. toxifera,* etc.), is macerated and triturated, boiled with a little water, and put into a tipiti press made from the jacitara palm (*Desmoncus macroacanthus*). This is allowed to exude slowly, then boiled to the consistency of an unguent and stored in little pots. Into this curare are dipped the tips of the slender darts made from the footstalks of the pataua palm (*Oenocarpus bataua*). These are used in blow pipes made from the paxiuba palm (*Iriartea setigera*). Curare contains two alkaloids: curarine, which paralyzes voluntary muscles by interrupting connection with the peripheral nerves at the motor end plates so that the animal lies helpless on the ground; and curine, which paralyzes the heart.

Certain Malay tribes use poisons from this genus which seem to contain strychnine and brucine as well as curarine, and may cause convulsions as well as paralysis. Other poisons used by certain Himalayan tribes have aconite as a base. *Hyoscyamus falezlez* (Solanaceae) has been employed by some of the natives in the interior of Africa and by the Tuaregs of the Sudan. Like ouabain it acts as a cardiac poison, although it contains hyoscyamine and scopolamine.

The sap and seeds of other species such as *Cerbera odollam* and *Thevetia nerifolia,* and in India the sap of *Antiaris toxicaria* have been used as arrow poisons, acting as powerful cardiac poisons.

Atriplicism. In North China poisoning has followed the ingestion of a weed, *Atriplex littoralis,* which grows in gardens around Pekin and which is sometimes eaten by the very poor in times of famine. It has been claimed that the poisoning is caused by a small

insect often present on the weed. About 15 hours after ingestion there appears itching of the fingers, quickly followed by swelling and discoloration. This swelling extends up the backs of the hands and outer surface of the forearms. The face also becomes swollen so that the eyelids may be closed, and the nose becomes cyanosed and cold. The swollen parts may ultimately develop blisters and ulcers. Later the finger tips may become gangrenous, as in Raynaud's disease. According to Yang (1940) symptoms do not appear unless the individual is exposed to bright sunlight, and as the lesions are limited to exposed areas, he and also Uyky regard the cutaneous lesions as a photosensitive dermatitis.

Cannabis Indica. This plant, Indian hemp, is the source of a serious drug addiction in Central Asia. In India the forms of the drug generally used are: "gangah," the dried flowering tops, which are smoked mixed with tobacco; "bhang," a mixture of the dried leaves and capsule, which is made into decoctions and is the cheapest form of the drug; and "charas," the resinous exudate obtained from the cut female heads of the plants. This latter is the most expensive and concentrated of the preparations. In Arabia a confection is made from charas and is known as "hashish." It is sometimes mixed with extracts of different Solanaceae such as datura and nux vomica, and in this form is said to be taken daily by millions of the inhabitants of Asia and Africa, although stringent regulations against its sale have been made in some localities. The drug gives a feeling of well-being, followed by hallucinations of sight and hearing, often of a sensual character. This is followed by dimness of vision, drowsiness, and stupor. Addicts may become insane.

Cannabis Sativa. The name "marihuana" has been given in Mexico and the United States to the flowering tops of this hemp plant, a member of the flax family which is widely cultivated in the United States and elsewhere for its fiber. It also grows wild as a weed. The flowering tops, especially those from the pistillate (female) plant, are smoked in India under the name "gangah." In some instances an extract is made from the tops and used to impregnate tobacco cigarettes. In the United States hemp cigarettes bear various other names, such as reefers, muggles, the weed, etc.

The intoxication, which may arise from smoking a single cigarette and may come on within about an hour, is characterized by excitement, mental confusion, talkativeness, and often spells of "hysterical" laughter. Visual hallucinations and sexual illusions are also features of the intoxication. Although euphoria is experienced, at the same time there is anxiety which may lead to ideas of suicide. Many of the patients treated in hospitals show psychoneurotic traits and the type of personality in which use of a drug for the pleasurable phases of its action is likely to be manifest. True addiction apparently does not occur, since neither an increase in tolerance nor withdrawal symptoms have been clearly demonstrated. In some cases, however, opium derivatives have been added in order to create an opium addiction.

There is general agreement that marihuana cigarette smoking brings about weakening of restraint and impairment of judgment, and it would seem that the excitation of sexual illusions might well lead to sex crimes or reckless indulgences. Many instances have been cited of homicidal attacks by persons when under the influence of marihuana. Bromberg (1939), however, in reporting his study of this addiction at Bellevue Hospital, questions whether it predisposes to crime, stating that no cases of murder or sexual crime were established as due to marihuana in 67 trials in the U. S. County Court of General Sessions.

The use of marihuana seems frequent in underworld resorts and has been reported among the better social classes. The dangers attending the use of the drug, especially by young people in the public schools, were first emphasized in New Orleans about 1926, and a federal law making the use of cannabis illegal was passed in 1937. In his book "Marihuana" (1938), Walton states: "The situation is of the utmost gravity and is one which calls for drastic measures of eradication."

Dermatitis Venenata. There are a great many plants in various parts of the world which cause various types of dermatitis—erythematous, vesicular, or urticarial. The best known of these plants belong to the Rhus family. In the United States by far the most common cause of plant dermatitis is poison ivy, *Rhus toxicodendron*, and the closely related variety called "poison oak." The poison sumac, *Rhus vernix*, in the north-central and northeastern states, and poison wood, *Metopium toxiferum*, in the extreme southeast, produce a similar, often more severe dermatitis. For poisoning to occur, there must be direct contact with the plant—the idea that a volatile poison is given off is erroneous. The plants seem more irritating when wet than dry, and a moist skin is more susceptible than a dry one.

The exciting agent is an oil, toxicodendrol, and in susceptible persons a minute amount ($\frac{1}{1000}$ mg.) can produce dermatitis. There are marked individual differences in reaction to these plants, and susceptibility is generally believed to be due to a form of allergic hypersensitiveness. Repeated attacks do not seem to confer immunity. Striking clinical improvement and diminished susceptibilty have been reported following treatment with a series of injections of Rhus extracts. The extracts are solutions in a vegetable oil of substances extracted from the fresh leaves of the plants.

Following exposure the skin should be scrubbed with soap and water. Alcoholic or oily solutions spread the inflammation.

In Japan the lacquer from *Rhus vernicifera* causes a cutaneous edema affecting the face and extremities, which is followed by a papular eruption.

In the United States there are many other plants capable of causing dermatitis. Among these are the parsnip (*Pastinaca sativa*), the lady slipper (Cypripedium), spurges (Euphorbia) and the primrose (Primula), particularly when in blossom. Lily rash is a dermatitis caused by handling various bulbs or stalks. Those handling the vanilla bean may suffer from an itching dermatitis. The rue group of plants is often responsible for skin irritation. Certain individuals may become specifically hypersensitive and react violently to plants which are harmless to the great majority of individuals.

Mangoes. Kirby Smith (1938) described a dermatitis that may follow eating mangoes. Burning and itching may appear six to eight hours after ingestion, affecting particularly the hands, neck, face and lips. The irritating resin is present in the skin or rind of the fruit and in the stem or sap, but not in the edible portion of the fruit.

Dhobie-mark Dermatitis. Livingood, Rogers, and Fitz-Hugh (1943) described 52 cases of dermatitis in American soldiers in India, which was caused by wearing clothes which had been marked with the juice of the marking nut from the *nal* or *bela gutti* tree. The dermatitis occurred only in the exact spots touched by the dhobie mark. The dermatitis recurred if marked clothes were again worn. Reaction to patch tests with the material were positive. Symptoms appeared within 8 to 24 hours and varied from a moderate erythema and edema to vesicular oozing and crusting lesions. (This must be distinguished from the "dhobie itch," frequently observed in the Philippine Islands, which is due to a fungus [trichophyton] infection.)

Goldsmith (1943) described a dermatitis resembling ivy poisoning in mail carriers and clerks in Washington, which was caused by handling mail which had been contaminated in the mail pouch with "bhilawanol oil" from the "Indian marking nut," *Semecarpus anacardium*. This plant, like Rhus and the mango, is a member of the family Anacardiaceae. Of about 50 individuals exposed, 16 were attacked. Undoubtedly other species of this family are capable of causing a similar dermatitis.

Favism. This is a disease characterized by an acute hemolytic anemia and caused by inhaling pollen from the flowers of the bean plant (*Vicia faba*) or by eating the bean. It is most frequent in Italy and especially in southern Sicily and Sardinia, where a morbidity rate as high as 5.17 per cent has been reported. Heredity seems to play a part, some families giving a history of favism over many generations. Ingestion of raw beans is more apt to cause it than eating cooked beans. This in connection with the

hypersensitiveness to the bloom indicates some type of allergic reaction. McCrae and Ellery (1933) reported a case in an Italian in Philadelphia who gave a positive cutaneous allergic reaction to an extract of the bean. In Italy, the attack comes on shortly after exposure with irregular fever and hemoglobinuria, and later pallor and jaundice. The abrupt and profound fall in red blood cells and hemoglobin (together probably with the associated renal injury) may cause death within a very short time. The mortality is about 5 per cent. Recovery, if it occurs, is complete.

Robinson (1941) reported six typical cases in Palestine following the eating of broad beans.

Ginger Paralysis. In 1930 an extensive outbreak of paralysis occurred in the south-central and southwestern portion of the United States, particularly in Tennessee and Cincinnati. It was largely restricted to adult males, all of whom had drunk Jamaica ginger from one-half to three weeks before the attack. It was characterized by a flaccid paralysis of the distal muscles of the legs which was preceded for three or four days by numbness and aching of the calves. In some of those afflicted the arms were subsequently involved. In some, death occurred, attributed to respiratory paralysis. In these cases examination showed degeneration of the myelin sheaths and axis cylinders of the radial, ulnar, sciatic, external popliteal, anterior and posterior tibial nerves. Carillo also reported degenerations in the spinal cord. The mortality was low, but disability in some cases persisted for two years and more. It has been estimated that from 10,000 to 15,000 cases occurred. It was later shown that the poisoning was due to contamination of the Jamaica ginger with about 2 per cent of triorthocresyl phosphate.

Similar poisoning has followed the use of apiol containing from 2[a] to 50 per cent of triorthocresyl phosphoric acid. Apiol is an alcoholic extract of the fruit of the common parsley (*Carum petroselinum* or *Apium sativum*). It has been used as an abortifacient, in menstrual disturbances and in malaria.

Hemlock. Poisoning from species of *Cicuta* (water hemlock, of the Parsley family) is not uncommon in man and cattle. The poison, cicutoxin, is a resinous substance found chiefly in the roots and root stalks. The leaves and fruit apparently may be eaten by animals without harm. Poisoning has occurred chiefly as a result of confusing the plant with parsnips or other edible roots. The symptoms are pain in the stomach, nausea, vomiting, diarrhea, dilated pupils, labored breathing, sometimes pulmonary edema, a feeble rapid pulse, and violent convulsions. Death may occur from respiratory failure.

The poison hemlock (*Conium maculatum*) is one of the most widely known poisonous plants because of its reputed use by the Greeks for the execution of State prisoners, notably Socrates. In recent times poisoning has occurred as a result of mistaking the seeds, leaves, or root for those of edible plants (anise, parsley, parsnips). It causes gradual progressive paralysis of the muscles, often loss of vision, and death from respiratory paralysis, without convulsions.

Jengkol Poisoning. In the Dutch East Indies poisoning has followed the ingestion of the fruit pods or beans of *Pithecolobium geminum*, a species of Mimosaceae. The fruit, which is said to have a high content of vitamin B, is eaten avidly by the natives in spite of its foul odor. It is sometimes put through an appetizing process (which increases its toxicity) by burying it in the ground for 10 days. When the beans begin to sprout they are taken up, ground, roasted. They contain ethereal oils which exert a powerful irritating action. If considerable is eaten, there may be severe colicky pains in the abdomen and flanks, as in renal colic, and vomiting, constipation, flatulence, and vesical tenesmus. The urine contains large amounts of blood and many small sharp crystals (jengcolic acid) which some believe injure the kidneys mechanically. There may be anuria, and death may follow, but recovery is the rule. The diagnosis is easily made from the characteristic disgusting odor of the breath and urine.

Kava or Yangona. An intoxicating nonalcoholic drink is made from the roots or leaves of the pepper plant, *Piper methysticum*, and is used as a ceremonial beverage in

many of the islands of the South Pacific. The parts of the plant are first chewed by young girls who have good teeth and good health, and the masticated material is put in a bowl and treated with cocoanut milk. A sort of quiet, drowsy intoxication with weakness of the legs results in those not habituated. In the chronic intoxication a condition of debility results, and a marked roughness of the skin is said to develop.

Lathyrism. This disease is characterized by the insidious onset of pains in the back and muscles and weakness of the legs, which gradually progresses to the development of a spastic paraplegia. There is wasting of the muscles and exaggeration of the reflexes, incontinence of urine and loss of sexual power. The arms are rarely involved. There are no sensory disturbances, and no mental or cardiac disturbances. Clinically lathyrism is practically indistinguishable from primary lateral sclerosis. It is rarely fatal, but runs a protracted course.

It is common in India, Abyssinia, and Algeria. Chopra (1940) emphasizes its frequency in India and states that examples of lathyrism in man in the form of spastic paralysis are commonly seen every day in the streets of Calcutta.

The disease has been attributed to eating bread made from the flour of the chick pea, *Lathyrus sativus,* or related vetches. In most of India this plant, known as Khesari dal, is an important article of food for both man and animals. Moderate amounts may be eaten with impunity, symptoms appearing only when large amounts are taken, especially to the exclusion of other foods. It has therefore been suggested that the syndrome is due to a deficiency of vitamin A, or possibly of tryptophane which is lacking in the Lathyrus seed proteins. There appears to be no direct evidence of a toxic substance in the peas. Acton and Chopra concluded that the disease is not due to Lathyrus but to a contaminating weed, *Vivia sativa,* from which a poisonous substance, divicine, has been obtained.

Minchin (1940) has reported similar cases from southern India where *Lathyrus sativus* is not eaten, but where the diets are grossly deficient in vitamins and proteins. He suggested that the syndrome is due to a deficiency of tryptophane, and therefore not directly dependent upon ingestion of this vetch. Although the question is manifestly unsettled, the theory of a dietary deficiency—probably a complex one—has great appeal and seems to conform best to the facts now known. There is abundant experimental evidence that degenerations of the nervous system can be produced in animals by a suitably deficient diet.

Manchineel Poisoning. The manchineel tree (*Hippomane mancinella,* of the Euphorbiaceae) has long been known as a source of poisoning in northern South America, Central America, the West Indies, and Florida. It is a large, handsome tree with holly-like or laurel-like leaves. The fruit, which resembles crabapples, has a pungent rather disagreeable taste but has sometimes been eaten with serious results. Hypersensitive individuals who handle the fruit may suffer from a dermatitis characterized at first by erythema and later by the formation of vesicles and bullae. The mucous membranes will become acutely inflamed if contaminated by the fibers. If the fruit is eaten, there may be swelling of the lips, blisters, and erosions of the buccal mucosa, dysphagia, nausea, vomiting, and diarrhea with blood in the stools. More rarely there may be profound collapse and death.

All parts of the tree appear to be toxic. There is a legend that death may result from sleeping under the shade of the tree. Raindrops falling on the skin of a person sheltered under it may cause irritation. The latex contains a greenish resin which is the toxic principle, and this has been used by the natives as a liniment. Smoke from the burning wood is said to cause severe conjunctivitis, and the sawdust may cause cough, rhinitis, laryngitis, conjunctivitis, and lacrimation. The dried fruit has been used as a diuretic, and the seeds contain a purgative oil.

Milk Sickness. Cattle eating richweed (white snakeroot, *Eupatorium ageratoides,* or *Urticae folium*) in the eastern United States or rayless goldenrod (*Aplopappus hetero-*

phyllus) in Texas acquire a serious disease called "trembles" or in some cases "alkali disease" or "milk sickness." In man, after ingestion of milk or butter from such a cow, there may appear anorexia, nausea, and vomiting which prevent the taking of food and water, and soon bring about an acidosis with a high mortality. The late symptoms are a subnormal temperature, an extremely low blood pressure, the presence of diacetic acid in the urine, and the odor of acetone in the breath and urine. The blood shows a marked ketosis, a lipemia, and a hypoglycemia. The poisonous principle, trematol, one of the higher alcohols, is found in the leaves and stems.

Mushroom Poisoning. The best known edible mushrooms are (in England and the United States) the meadow mushroom, *Agaricus campester,* which grows only in open pastures, and (in France and Italy) the champignon, *Marasmius oreades.* There are no criteria by means of which edible mushrooms can be distinguished from poisonous varieties. There is a widespread entirely erroneous belief that if a silver coin is put in the dish in which mushrooms are cooked, it will be tarnished if poisonous varieties are present. Poisonous mushrooms cannot be recognized by the taste—the most poisonous species are said to have a very agreeable flavor. In Washington an Italian officer who was regarded as an expert in the recognition of edible species purchased in a market some mushrooms which had been collected in nearby Virginia. He breakfasted on these mushrooms and spoke of their fine flavor. In about 15 minutes he became acutely ill, developed blindness, dysphagia, and convulsions and died within 24 hours. If one has not the expert knowledge required to identify the species with certainty, one should eat only mushrooms which have been passed upon by a competent (preferably official) inspection. Even in cultivated beds poisonous species occasionally develop.

There is marked individual variation in susceptibility. In the case of a family of six poisoned by *Amanita nappa,* reported by Bentkowski, four died (one of whom had eaten only a mouthful); one became ill but recovered, and the sixth who had eaten heartily suffered no ill effects.

In the United States most cases of poisoning are due either to *Amanita muscaria,* the fly amanita, or to *A. phalloides,* the death cup. Both species are very common and widely distributed. One feature which helps to identify this genus is the persistence of a portion of the veil encircling the stem a little below the cap.

The poison in *A. muscaria* is muscarin, an alkaloid related to pilocarpin. This type is distinguished by the early appearance of symptoms (within three hours), and death in the fatal cases occurs within 24 hours. There are nausea, vomiting, diarrhea, severe abdominal pain, sweating, salivation, lacrimation, miosis, often a slow irregular pulse, and in fatal cases convulsions and coma. Atropin is an efficient antidote, and although the symptoms are violent, the mortality is low in properly treated cases (about 10 per cent).

The poison in *A. phalloides* is a toxin. In poisoning of this type the symptoms are late in appearing (6 to 18 hours), and although the mortality is from 50 to 70 per cent, death usually occurs only after five to eight days. In addition to the gastrointestinal symptoms noted above, there may be great thirst, anuria, jaundice (after two or three days), cyanosis, drowsiness, delirium, or coma. There is degeneration of the renal tubular epithelium and liver necrosis. Extensive degeneration of the ganglion cells of the cerebral cortex, basal ganglia, cerebellum, and brain stem have been described (Vander Veer and Farley, 1935). Atropin has no effect. An antitoxin has been used in Europe with alleged good results.

Another rarer type of mushroom poisoning characterized by an acute hemolytic anemia, hemoglobinuria, and jaundice has been described. Recovery is the rule.

Mustard-oil Poisoning (Epidemic Dropsy). This disease resembles beriberi in that it is characterized by dropsy associated with myocardial disturbances without anesthesia or marked paralysis. The disease has been recognized in India (Calcutta) since 1877. Outbreaks have recurred at intervals since 1913, one of the worst in 1926, when over

2000 cases were observed. In an outbreak in Mauritius in 1879 one-tenth of the coolies were attacked and a large number died. In 1926 in Fiji an outbreak occurred which was confined entirely to the Indian population. This suggested that the poisoning was caused by mustard oil used in preparing curries. In subsequent experiments on human volunteers in the Calcutta jail, characteristic symptoms were produced by feeding the suspected oil. Chopra and Badhwar (1940) pointed out that in some of the epidemics the mustard oil was adulterated with katakar oil from the seeds of *Argemone mexicana,* the Mexican poppy or shialkata, which superficially resemble mustard seeds. In further experiments in human beings they produced typical disease symptoms by administering food cooked in oil containing controlled amounts of Argemone oil. Lal et al. (1941) reported the isolation from the Argemone oil of a white crystalline basic substance which seems to be the toxic principle. Rats fed the purified mustard oil suffered no ill effects. The exact nature of the substance was not reported.

Opium. The use of opium is common among the inhabitants of India and the Orient, and there are many addicts in Europe and America. In India, opium is almost invariably taken in pill form, and when used in this way the ill effects are much reduced. The mental, moral, and physical deterioration so common in those who smoke opium, as in China and Persia, or in those who use morphine or other alkaloids hypodermically, is not so marked. There is also less tendency to increase the dose. Every medical man should read De Quincey's "Confessions of an English Opium Eater" to appreciate the slight effect this habit had on the author during the first few years of his addiction. He took the drug as did the native of India. In the Far East, native mothers and wet nurses sometimes smear the nipples of the breast with the drug, and it has been a not uncommon custom for the native nurses or ayahs to soothe babies to sleep by dipping their fingers in opium and allowing the babies to suck upon these fingers. Such treatment is obviously highly deleterious to the child. The minute contracted pupils may suggest this form of intoxication in cases of obscure illness.

Miscellaneous Sources of Plant Poisoning. In addition to the diseases described, numerous other instances of poisoning by plants are known, some accidental in origin and some intentional. Chestnut (1938) listed only about 30 species of plants associated with accidental poisoning in man, usually the result of confusing poisonous and harmless species. Muenscher (1939), however, listed 400 species of plants in the United States which have caused poisoning either in man or animals. The families which contain the largest number of poisonous species are the Liliaceae, Ranunculaceae, Leguminosae, Euphorbiaceae, Umbelliferae, Solanaceae, and Compositae.

Prussic acid occurs in many valuable foods, and if not removed may cause serious poisoning and even death. The linseed (flax) plant, *Linum usitatissimum,* contains a cyanogenetic glucoside which occurs early in the development of the plant and persists in the seeds.

Mandioca Poisoning. The roots of the plants *Manihot aipi,* the sweet cassava, and *Manihot utilissima,* the bitter cassava, constitute one of the most important articles of diet in many parts of Africa, South America, and the West Indies. The roots are generally dried and ground into a powder and used as a flour for making cassava cakes. In more civilized areas they are used to produce starch and tapioca. Bitter cassava contains a glucoside which liberates hydrocyanic acid in the presence of water and causes serious poisoning if eaten without preparation. To avoid poisoning, the tuber must be scraped, grated, squeezed free of its milky juice, and thoroughly washed. It is then often dried in the sun.

According to Holland (1938) the ripe fruit of the cultivated tree *Pangium edule,* in New Guinea, is wholesome. The unripe fruit, however, and the fruit of the wild tree at all stages are highly poisonous and cause serious illness and even death. The kernels contain a large amount of hydrocyanic acid, probably in combination as a glucoside. The scraped kernels are said to have a sweet cocoanut-like taste and cannot be detected

when added to other food. They have been often used for homicidal purposes. The chief use of the nuts in some villages has been in stealing fowls. The scraped kernels are thrown to fowls which die quickly after eating them. If the crop is then removed, the fowl may be eaten.

Oxalic acid is contained in variable quantity in many edible plants, as sour grass and rhubarb, and may cause poisoning if ingested in sufficient quantity.

Various species of *Jatropha*, physic nuts, found in India and the West Indies, cause severe diarrhea and gastrointestinal irritation similar to that caused by croton oil. A similar poisoning in Tanganyika has been reported as due to eating the nuts of the coral plant.

The consumption of unripe persimmons, usually by children, has resulted in the accumulation of a mass of pulp in the stomach, forming a bezoar similar to the well-known hair balls. These may become large and require operation for removal.

Plants Used Especially for Homicidal or Suicidal Purposes. The root of *Gloriosa superba*, sometimes called wild aconite, a plant widely distributed in tropical Asia and Africa, has been used as a poison. The toxic effects which are due to the active principle, "superbine," resemble those of aconite poisoning—tingling and numbness of the lips and pharynx, nausea, vomiting, abdominal pain, cardiac depression, and collapse with terminal convulsions. Consciousness is usually retained.

The roots of various species of *Aconitum* have been used for the same purpose, death often taking place in from three to four hours.

Various plants belonging to the order Solanaceae are used in many parts of the Tropics and by criminals in temperate climates to produce unconsciousness. Chopra has reported fatal cases of poisoning from *Jatropha* in India, in which the symptoms were dryness of the mouth and throat, dilatation of the pupils, and delirium.

The seeds of *Datura fastuosa* are used by the Thugs in India, and various other plants whose alkaloids have an action similar to belladonna are used by the natives of many parts of the Tropics. The seeds of datura have only a slight taste, and consequently are easily introduced into food. *D. sanguinea* has been employed in Peru and Colombia, and *D. ferox* and *D. arborea* in Brazil. The characteristic seeds are sometimes found in the feces or in fatal cases in the intestine.

The dried leaves of *Hyoscyamus niger*, henbane, are the basis of some of the "knock-out" drops used by the underworld. In poisoning from these plants the face is flushed, the pupils widely dilated, the eyes bright and shining. The throat is very dry. There is marked disturbance of vision. At first the victim is very talkative and soon becomes violent, but later there is drowsiness followed by coma.

The yellow oleander, *Cerbera thevetia*, which is found in India, contains highly poisonous glucosides, nereoside, and oleandroside, in the milky latex. Similar substances from *Urechites suberecta*, urechitin and urechitoxin, possess a cumulative action, and sudden death may occur before suspicion of poisoning is aroused.

In New Guinea, Holland (1928) reported that eating the roots of wild species of *Derris* is the commonest means of suicide among the natives. The root is known as "bun," and it was identified as Derris root only after the plant became of value as an insecticide. The poisonous substance of *Derris eliptica* is rotenone. Other toxic resins, such as derride, are also present. The conditions Holland found at autopsy are indicative of acute congestive heart failure. As an antidote the natives use the sap expressed from the roots of the banana, which is mucilaginous and acts as an emetic.

The juice of a species of *Asclepias* (Milkweed family) has been used in India as an infanticide. The symptoms of poisoning are salivation, vomiting, cramps, and final collapse. Common native poisons employed in Brazil are prepared from *Paullinia pinnata* which contains an alkaloid timboin, and from the fruit of *Thevetia ahonai* which contains the poison thevetosin. Both of these cause vomiting and acute respiratory failure. In the Dutch East Indies the common poison is extracted from the roots of species of

Milletia. Following its ingestion, debilitation, headache, diarrhea, and collapse usually occur, followed by death.

H. Laboratory Procedures Useful in Diagnosis, Indexed by Diseases

This index has been compiled to assist in the selection of laboratory procedures which are likely to be of value in the diagnosis of the diseases listed, particularly those which cannot be recognized by their clinical features alone. Only those of major importance can be given. The statements in the index apply to the average case, and many are subject to limitations or qualifications for which the text should be consulted. To be of value, laboratory tests must be performed with such precision that the probable technical error is well within the limits of the physiologic variation. The results can be interpreted correctly only in conjunction with all the information available concerning the patient. This responsibility belongs to the clinician, and should not be expected of, or entrusted to a laboratory technician.

Abscess. Aspirate contents with sterile pipet or syringe and culture on agar and blood agar plates. Stain films by Gram's method and, if indicated, for acid-fast bacilli. In special cases inoculate animals. Usually a neutrophilic leukocytosis. Sedimentation rate increased.

Abscess of Liver, Amebic. Aspirate contents aseptically and culture on blood agar plates (no growth unless secondarily infected). Pus resembles anchovy sauce. Stained films usually show detritus with few pus cells. Amebae found in fresh preparations only after drainage has been established. Examine feces for trophozoites and cysts. Moderate neutrophilic leukocytosis if acute. Monocytosis. Roentgenogram.

Abscess, Lung. Sputum abundant, purulent, often foul; may layer on standing. Culture aerobically and anaerobically. Stain by Gram and Ziehl-Neelsen methods and examine *fresh* material for spirochetes and fusiform bacilli, preferably with darkfield. Look for elastic fibers. Exploratory aspiration in some cases. Leukocytosis. Roentgenogram. Bronchoscopy. Differentiate from tuberculosis, fungus infections, ruptured liver abscess, tumor.

Abscess, Tuberculous. If stained films of the pus are negative for pyogenic organisms, stain for acid-fast bacilli. Concentrate with alkali if necessary. Make cultures and inoculate a guinea pig. Leukocyte count variable. Sedimentation rate increased.

Achylia Gastrica. Do fractional gastric analysis after histamine injection. (See p. 861.)

Acidosis. If possible test CO_2 combining power of plasma. Alternatives: Test CO_2 tension of alveolar air. Measure titratable acidity + ammonia output in urine. Determine tolerance for $NaHCO_3$. Look for ketone bodies in urine. Test pH of urine. Ammonia in urine increased at expense of urea. (See pp. 802 and 840.)

Actinomycosis. Look for yellow "sulfur" granules in pus or sputum. Press out granules between slides and stain by Gram's method. The central mycelium is Gram-positive while peripheral "clubs" are Gram-negative. Culture anaerobically. (See p. 89.)

Acute Yellow Atrophy of Liver. (See Liver, necrosis of.)

Addison's Disease. In crises, marked reduction in serum sodium and total base, and in chlorides and bicarbonate. High blood nonprotein nitrogen and urea. Hypoglycemia. "Flat" glucose tolerance curve. During remissions salt restriction may precipitate a crisis (dangerous). Hypersensitive to insulin injections. Roentgenogram for calcified areas in adrenals.

Agranulocytosis. See Malignant neutropenia.

Alkalosis. Test CO_2 combining power of plasma. Test pH and titratable acidity of urine. Look for signs of tetany.

Allergy. See Hypersensitiveness.

Amebiasis. Note gross and microscopic appearance of the stools (see p. 535). Examine feces for trophozoites and cysts. May show monocytosis. Differentiate from bacillary dysentery and chronic (nonspecific) ulcerative colitis.

Amyloidosis. For changes in blood and urine see "Nephrosis." Make Congo red test (p. 831). Search for evidences of chronic suppuration or tuberculosis.

Anaphylaxis. See Hypersensitiveness.

Ancylostomiasis. Examine feces by concentration methods for characteristic ova (see p. 641). Examine blood for anemia (hypochromic, microcytic) and eosinophilia.

Anemia, Aplastic. (See p. 442.) Qualitative changes in red cells slight. No signs of regeneration. Marked neutrophilic leukopenia and thrombocytopenia with purpura. Sternal marrow shows few erythroblasts or myelocytes.

Anemia, Hemolytic. (See p. 445.) Positive indirect van den Bergh. High icterus index. Urobilin increased in urine and feces. Reticulocytes usually increased. Leukocytosis. In fulminant cases look for hemoglobinuria.

Anemia, Hypochromic. (See p. 432.) Cells very pale, usually small. Color index and saturation index low. Corpuscular volume usually low. Icterus index normal or low.

Anemia, Idiopathic Hypochromic. Largely limited to women 20 to 50 years old. Usual features of hypochromic anemias marked. Leukopenia frequent. Subacidity or achlorhydria, usually even after histamine. Reticulocyte crisis follows administration of large doses of iron. Differentiate from chronic posthemorrhagic anemia, chlorosis, cancer, hookworm anemia. (See p. 433.)

Anemia, Pernicious. (See p. 437.) High volume index and high color index. Marked anisocytosis, macrocytosis, poikilocytosis. Megaloblasts in severe untreated cases. Evidences of hemolysis during progressive stages. Leukopenia with hypersegmented neutrophils. Blood cholesterol reduced. Achlorhydria after histamine. Reticulocyte crisis follows administration of potent liver extract in severe cases.

Anemia, Posthemorrhagic, Acute. Features of hypochromic anemia present in moderate degree. Usually reticulocytosis, leukocytosis, and increase in platelets. Normoblasts may appear after third day.

Anemia, Posthemorrhagic, Chronic. Features of hypochromic anemia marked. Low color and saturation index. Evidences of regeneration variable. In late stage may show aplastic type of anemia. (See p. 432.)

Anemia, Sickle Cell. Seal fresh moist preparations and observe for 1 to 24 hours for sickled red cells. (Rare in stained films.) Severe cases show many normoblasts, reticulocytosis, leukocytosis, increased platelets. Look for macrophages containing red cells. Roentgenograms of skull may show changes. Limited to Negroes.

Angina, Streptococcal. Make cultures on Loeffler's serum and blood agar plates. Stain films with Loeffler's methylene blue and Gram's stain. Differentiate from diphtheria and Vincent's angina.

Angina, Vincent's. See Fusospirochetosis.

Anthrax. (1) Malignant pustule. Examine material from pustule directly in stained films and hanging drop for large, Gram-positive, nonmotile bacilli. Culture on agar. Inoculate a mouse or guinea pig subcutaneously. Blood cultures rarely positive. (2) Woolsorters' disease. Examine sputum in a similar way.

Appendicitis. Neutrophilic leukocytosis with increase in juvenile forms. Differentiate from renal and pelvic infections.

Arsenic Poisoning. Examine urine for arsenic (in the late stages, the hair). Examine urine for albumin, casts, renal epithelium, urobilin, bile. Examine blood for nonprotein nitrogen or urea, creatinine, bilirubin. Test renal function and liver function. Test for acidosis.

Arthritis, Acute. Neutrophilic leukocytosis. Make blood culture. In special cases aspirate fluid, culture on blood agar, and stain films by Gram's method. Look especially for streptococcus, gonococcus, meningococcus, and brucella. Also streptobacillus.

Arthritis, Chronic Infectious. Joint fluid usually sterile. (See p. 761.) Antifibrinolysin and antistreptolysin titers may be of help. Streptococci can sometimes be cultivated from excised lymph nodes draining affected joints. Use deep tubes of serum glucose agar. Sedimentation rate increased. Occasional leukocytosis. Blood uric acid normal.

Ascaris Infection. Examine feces for characteristic ova. Worms occasionally found in feces or vomitus.

Ascites. Culture fluid on blood agar. Determine whether transudate or exudate. (See p. 760.) If tuberculosis is suspected digest sediment with alkali, and examine centrifugate for acid-fast bacilli by staining, special cultures, and guinea-pig inoculation. See also cirrhosis of the liver, edema, nephritis.

Asthma. Examine sputum for eosinophils, Charcot-Leyden crystals, Curschmann's spirals. Make culture from sputum for possible causative organism in cases due to respiratory infection. Eosinophilia. Make cutaneous allergic tests. Severe cases with cyanosis show decreased O-saturation in arterial blood. May show acidosis (CO_2 excess) with high plasma bicarbonate.

Bacteremia. Make blood culture. Usually neutrophilic leukocytosis with shift to the left.

Bacteriuria. See Urinary-tract infections.

Balantidium Infection. Look for large motile ciliates in feces.

Banti's Disease. Moderate to severe anemia, usually hypochromic in type. Leukopenia. Test fragility of the red cells to exclude hemolytic jaundice. Make van den Bergh test and determine icterus index. Test liver function. Examine feces for occult blood.

Blackwater Fever. Hemoglobinuria; pink foam to urine; test filtrate for hemoglobin spectroscopically and by benzidine or orthotolidine test. Malarial parasites found in thick blood films in rare cases. Leukopenia. Monocytosis. Examine blood serum for hemoglobin and bilirubin (van den Bergh test). Reaction to Donath-Landsteiner test negative.

Blastomycosis. Examine sputum, pus, or scrapings from margins of ulcers in 10 per cent KOH for spherical, budding yeast cells with highly refractile, double-contoured walls. Make culture on glucose agar plates.

Botulism. Inject an infusion of the suspected food into a guinea pig (see p. 72); also stools and blood of patient. Culture anaerobically on glucose agar. Culture may be kept in a dark place at room temperature.

Bronchiectasis. Sputum abundant, purulent, often foul, tends to separate into three layers on standing. Small hemoptyses common.

Bronchitis. Culture sputum on blood agar plates. Stain film by Gram's method. In special cases inoculate a mouse.

Bronchopneumonia. Culture sputum on blood agar. Make blood culture.

Brucellosis. Make blood culture at onset of febrile paroxysm; incubate in atmosphere of 10 per cent CO_2. Make cultures from urine, feces, and local foci in special cases. Guinea-pig inoculation sometimes successful. After fifth day make agglutination tests. Intradermal tests, an opsonic index. Relative lymphocytosis, often leukopenia. Differentiate from typhoid fever, tuberculosis, malaria, kala-azar.

Carbon-monoxide Poisoning. Test blood for CO spectroscopically and by Sayers-Yant method. Secure blood at earliest possible moment and protect from air.

Cerebrospinal Fever. See Meningitis, meningococcus.

Cestode Infections. Examine feces for ova, which are not always present. If a segment is obtained, press between two glass slides and examine the branchings of the uterus.

Chancroid—Ducrey's Bacillus. Examine smears for short, Gram-negative coccobacilli occurring in chains. Culture material aspirated from bubo in sterile clotted human or rabbit blood which has been inactivated at 56° C. for 30 minutes. Skin test with vaccine. Syringe and media must be warm.

Chlorosis. Hypochromic microcytic anemia, sometimes severe, in adolescent girls. Low saturation index. Gastric acidity normal. Differentiate from chronic posthemorrhagic anemia.

Cholecystitis. May be moderate leukocytosis. Fluid obtained by duodenal drainage may show bile-stained pus cells and other abnormalities. (See p. 868.) Culture on blood agar plates and enteric media. Van den Bergh test.

Cholelithiasis. Examine blood and urine for bilirubin (van den Bergh test). Bile obtained by duodenal drainage often shows increase in cholesterin crystals and calcium bilirubinate precipitate, and evidences of cholecystitis (q.v.). Blood cholesterol increased, with obstructive jaundice. Roentgenogram (Graham test).

Cholera. Smears from flecks in rice-water stools show many vibrios with "fish-in-stream" arrangement. Culture on Dieudonné plates. If sparse, use enrichment method. Identify organism with cholera immune serum. After fourth day test serum for agglutinins. Intense dehydration with high blood counts, high plasma proteins, and high specific gravity of blood. Anuria with high nonprotein blood nitrogen. Acidosis from loss of base. Depletion of chlorides. Differentiate from food poisonings, arsenic or antimony poisoning, bacillary dysentery, algid pernicious malaria.

Chyluria. Centrifuge urine and examine for microfilariae. Examine blood for microfilariae (not always present). Urine contains many highly refractile fat globules soluble in ether.

Cirrhosis of Liver. Do Kolmer or Kahn test. Icterus index and van den Bergh test. Test urine for bilirubin and urobilin. Make tests of liver function (p. 874) or determine A/G ratio. Examine feces for occult blood. Late cases may show macrocytic anemia. (See also Ascites; Banti's disease; Liver, necrosis of.)

Coccidioidomycosis. Examine fresh moist specimen of sputum, cleared with 10 per cent KOH for spherical yeastlike cells which may contain endospores. Culture and inoculate animals if necessary. Make intracutaneous tests, and complement-fixation test if possible. If ulcers are present, examine scrapings in a similar way. Biopsy if necessary. Make blood culture. Exclude tuberculosis by repeated stains, cultures and guinea pig inoculation. (See p. 235.)

Colitis, Chronic Ulcerative. Examine feces or preferably scrapings from ulcers (proctoscope) for pus, blood, mucus. Exclude amebic and bacillary dysentery by fresh warm-stage preparations, cultures, and agglutination tests. Neutrophilic leukocytosis, often secondary anemia, increased sedimentation rate. Roentgenograms.

Colitis, Mucous ("Spastic Colitis"). Examine feces for mucus in large masses, containing epithelial cells, often eosinophils, no pus cells, no blood. No leukocytosis, normal sedimentation rate. Roentgenograms.

Coma. Examine urine for sugar, ketone bodies, albumin, casts, blood. Examine blood for sugar, CO_2 combining power, nonprotein nitrogen or urea, and in special cases for alcohol and CO. Consider possibility of other poisons. Examine spinal fluid (caution, see p. 746), especially for pressure, presence of red cells and xanthochromia (subarachnoid hemorrhage). Make leukocyte count and blood culture if febrile.

Conjunctivitis. Stain smear by Gram's method and with dilute carbolfuchsin. Culture secretion on blood agar and plain agar. (See p. 731.)

Coronary Thrombosis. Neutrophilic leukocytosis. Accelerated sedimentation rate.

Cyst. Examine fluid for echinococcus hooklets and scolices. Test for pancreatic ferments and for urea.

Cystitis. See Urinary tract infections.

Dehydration. Examine blood for increase in hemoglobin concentration and in plasma protein (the best gauge). Specific gravity is increased.

Dengue. Neutrophilic leukopenia.

Dermatophytoses. Examine scrapings from skin in 10 per cent KOH for fungi. Culture on Sabouraud agar.

Diabetes Insipidus. No albuminuria or glycosuria. No nitrogen retention. Ability to concentrate urine lost, restored temporarily by pituitrin injections.

Diabetes Mellitus. Examine urine for sugar and ketone bodies. Examine blood (fasting) for sugar, cholesterol. In doubtful cases do glucose tolerance test. Make tests for acidosis (q.v.). In severe cases test for decrease in blood bases (or chlorides) and for dehydration.

Diphtheria. Make smears and cultures on Loeffler's serum or whole-egg medium. Stain by Gram's and Loeffler's or Neisser's method. Look for parallel rods containing polar granules. In special cases isolate on tellurite blood agar and inject guinea pig with broth culture filtrate as test for virulence. Make Schick test on contacts. Differentiate from streptococcus and Vincent's infections.

Diphyllobothrium Infection. Operculated ova in feces. If segments are obtained, press one between two glass slides and look for characteristic rosette-shaped uterus. Macrocytic anemia occurs in very rare instances.

Dracunculus Infection. Moisten blister or ulcer with a few ml. of water. Examine fluid excreted by worm for striated larvae.

Duodenal Ulcer. Make gastric analysis (for hyperacidity, blood, hypermotility). Examine feces for blood. Roentgenograms.

Dysentery, Amebic. Examine mucus from fresh warm stool (warm stage) for amebae actively putting forth bladelike pseudopodia. If necessary pass rectal tube, give saline purge, or scrape base of ulcer through a proctoscope. Pathogenic amebae often contain red cells. Examine feces for cysts. Smear of feces shows granular detritus, often Charcot-Leyden crystals, few pus cells. Monocytosis. No eosinophilia.

Dysentery, Bacillary. The sanguinolent mucus contains many pus cells and many phagocytic endothelial cells. Emulsify mucus in sterile broth or salt solution and plate on S. S. and MacConkey media. Identify organisms isolated by agglutination with immune sera. Neutrophilic leukocytosis. After 7 to 10 days make agglutination tests.

Echinococcus Disease. Examine fluid from cyst for hooklets. Eosinophilia. Make complement-fixation, precipitin, or cutaneous tests with special antigen (see p. 649). Roentgenograms.

Edema. Examine urine for evidence of nephritis. Test renal function. Determine plasma proteins, A/G ratio, blood chlorides. (See Nephrosis.)

Elephantiasis. See Filariasis.

Empyema. Neutrophilic leukocytosis. Make Gram's stain of smear from pus. Culture on blood agar plates. Roentgenogram.

Encephalitis, Epidemic. Examine spinal fluid. (See p. 201.) Make cultures and stains of sediment to exclude ordinary types of meningitis.

Endocarditis, Subacute Bacterial. Make blood culture (repeatedly if negative). (Plates usually show *S. viridans,* rarely pneumococcus, gonococcus, or Pfeiffer bacillus.) Moderate neutrophilic leukocytosis (inconstant), often increase in macrophages. Progressive anemia. Increased sedimentation rate. Examine urine for albumin, casts, blood (renal emboli).

Enteritis, Tuberculous. Stain smear from feces for acid-fast bacilli. Inoculate guinea pigs. There are often present in feces acid-fast, sporelike bodies which should not be mistaken for tubercle bacilli. Tubercle bacilli may be present in feces when there is no involvement of intestine (swallowed sputum, particularly in children).

Eosinophilia. Look for ova of intestinal parasites in feces. Look for evidence of trichinosis, hydatid disease, filariasis, various skin diseases, asthma and other allergic reactions, and lesions of the bone marrow.

Erythremia. See Polycythemia.

Espundia (Mucocutaneous Leishmaniasis). Examine scrapings from base of ulcer for leishmanial forms. Stain with Wright or Giemsa. Preferably obtain material by aspiration by puncture near edge of ulcer. If bacterial contamination can be avoided, culture on special media. Obtain material from adjacent lymph nodes by puncture.

Exophthalmic Goiter. High basal metabolic rate. Low blood cholesterol, high sugar. Glucose tolerance curve shows high peak, often prolonged. Glycosuria common. Often lymphocytosis.

Favus. Place hair or portion of favus cup on a slide and examine in 10 per cent KOH for mycelium and spores which are very irregular.

Filariasis. Examine blood (day and night) for microfilariae, either in fresh moist preparation or better in thick films stained by Giemsa. (In elephantiasis microfilariae are often absent from blood.) Aspirate lymph varix or hydrocele and examine sediment for microfilariae. Examine sediment of chylous urine or ascitic fluid. Often eosinophilia. Complement-fixation test positive with special antigen.

Flagellates, Intestinal. Examine feces emulsified in salt solution for motile flagellates. Emulsify another portion in Gram's solution to study flagella. Stain smear with iron hematoxylin for encysted forms. (See pp. 541 and 548.)

Furunculosis. Examine stained film and culture on blood agar. Determine blood sugar, or preferably make glucose-tolerance test.

Fusospirochetosis (Vincent's Angina and Stomatitis). Stain films of material from the depths of the lesion with dilute carbolfuchsin or Fontana's stain. (The spirochetes and fusiform bacilli are present in large numbers; a few may be present in normal mouths.) Make cultures on Loeffler's serum and blood agar to exclude diphtheria and streptococcal infection. Examine blood to exclude leukemia and agranulocytic angina. There may be a marked lymphocytosis. Exclude syphilis by Kolmer or Kahn test. In pulmonary infections examine *perfectly fresh* sputum in stained films and darkfield preparations. (See Abscess of lung.)

Gas Gangrene. (Infections are usually mixed; important organisms are *Cl. perfringens, Cl. oedematiens* and *Cl. oedematis-maligni.* Precise identification difficult but important because of serum treatment.) Make hanging drop and stain smears from exudate by Gram's method and for capsules. (All are Gram-positive; *Cl. perfringens* is encapsulated and nonmotile; the other two are motile and nonencapsulated.) Make anaerobic cultures in milk, glucose agar, and meat tubes. (*Cl. perfringens* causes gas formation and disruption of the tube overnight.) Blood cultures (anaerobic) may be positive. (For isolation and identification by inoculation into immunized animals see p. 67.)

Gastric Cancer. Examine fasting stomach contents for retention of food, lactic acid, Boas-Oppler bacilli, occult blood. Test meal shows achlorhydria and delayed motility. Occult blood in feces. Usually anemia, hypochromic microcytic; often neutrophilic leukocytosis. Increased sedimentation rate. Roentgenogram.

Gastric Ulcer. Examine fasting stomach contents for free HCl, food retention, sarcinae, blood. Test meal shows normal or high acid, often delayed emptying. Occult blood in feces, often intermittent. Roentgenogram.

General Paresis. Examine spinal fluid: shows increased cell count, positive globulin test, paretic colloidal gold curve, positive reactions to Kolmer and Kahn tests. Blood shows positive reactions to Kolmer and Kahn tests.

Glanders. Smears from pus show characteristic Gram-negative bacilli, parallel beaded rods. Culture on acid glycerin agar and potato. Inoculate male guinea pig intraperitoneally (see p. 121). Complement-fixation and agglutination reactions may be positive. (Mallein tests in animals.)

Glycosuria. Examine urine for sugar and apply special tests for glucose. Fasting blood sugar and glucose-tolerance test. Consider renal diabetes and other causes for glycosuria. (See pp. 774 and 779.)

Gonococcus Infection. Gram's stain of smear from urethra, cervical canal, or eye shows intracellular, Gram-negative diplococci. Make culture. (See p. 49.) Complement-fixation test useful in chronic infections.

Gout. Uric acid in blood often increased. Examine serum or plasma. Examine crystals curetted from tophus, if present, by murexide test.

Granuloma Inguinale. Stain scrapings from ulcers for Donovan bodies in large mononuclear cells (Wright or Giemsa).

Hematuria. Examine sediment for red cells. Apply orthotolidine test. (For causes see p. 82.)

Hematuria, Egyptian. See Schistosomiasis.

Hemochromatosis. Examine urine for sugar and for hemosiderin granules in sediment. Fasting blood sugar or glucose-tolerance test if in doubt. Make van den Bergh test. Test liver function. Examine piece of excised skin for hemosiderin.

Hemoglobinuria. Examine centrifuged sediment for intact red cells. "Shadow cells" may be found with much debris. Test filtered urine for hemoglobin spectroscopically and by benzidine or orthotolidine test.

Hemophilia. Prolonged coagulation time. Normal bleeding time, normal platelet count, normal clot retraction. Negative reaction to tourniquet test. May show post-hemorrhagic anemia. (A congenital abnormality limited to males.) Roentgenograms of joints in selected cases.

Hepatitis. See Jaundice, catarrhal.

Hodgkin's Disease. Examine section of excised lymph gland. The blood may show moderate leukocytosis, monocytosis (10 per cent to 15 per cent), increased platelet count, occasionally eosinophilia (no constant changes). In late stages, a severe anemia. Biopsy of marrow in special cases. Roentgenogram of chest.

Hookworm Infection. See Ancylostomiasis.

Hypersensitiveness. Test for hypersensitiveness to pollens, animal hair, foods, etc., by cutaneous tests or intracutaneous injections of suitable extracts, or in special cases by patch tests. To determine if a patient is sensitized to a serum, inject intradermally 0.1 ml. of a 1 : 10 dilution of the serum. If he is sensitized, an urticarial wheal will develop within 10 or 15 minutes. A drop of serum in 1 : 10 dilution may be instilled into the conjunctiva (see p. 295).

Hypertension, Essential. Examine urine and test renal function, especially by concentration tests, to determine extent to which kidney is involved.

Infectious Mononucleosis. Leukocytosis with high lymphocytosis (up to 90 per cent). Many pathologic lymphocytes. (May not appear until several days after onset and may persist for months.) After 5 to 10 days test serum for "heterophil" agglutinins for sheep red cells.

Influenza. Leukopenia and granulocytopenia. Frequent secondary infections, particularly of the respiratory tract.

Intestinal Parasites. Examine feces for ova.

Jaundice, Catarrhal (Infectious Hepatitis). Test urine for bile. High icterus index. Positive van den Bergh reaction; type variable. (See p. 872.) Feces show diminished or absent bile pigments, increased fat. Test liver function. Differentiate from Weil's disease, arsenic and phosphorus poisoning, types of obstructive jaundice.

Jaundice, Familial Hemolytic. Usual features of hemolytic anemia marked. Fragility of red cells in hypotonic salt solution increased. Reticulocytes much increased. Red cells have normal volume but diameter is small (more globular than normal). Look for evidences of cholelithiasis.

Jaundice, Infectious (Weil's Disease). Examine blood (first three days) for leptospira in stained films or darkfield preparations (sparse). Preferably inoculate a guinea pig with 1 to 5 ml. of blood intraperitoneally and at autopsy examine the liver for leptospira. Blood cultures may be made in Fletcher's medium and incubated at 25° to 30° C. After the twelfth day examine urine sediment for leptospira by smear and guinea-pig inoculation. If stock cultures are available, after 10 to 14 days patient's serum may give a positive adhesion phenomenon or agglutination or protection test. Leukocytosis.

Jaundice, Obstructive. Blood shows high icterus index and positive direct van den Bergh tests, increase in cholesterol and often in diastase. Prothrombin time increased. Urine shows bilirubin, no urobilin. Feces show increased fat and (if obstruction is complete) no bilirubin or urobilin. Fragility of red cells normal or diminished. Liver function may be reduced in chronic cases.

Kala-azar. Culture blood. Search for *Leishmania* within the leukocytes and monocytes in blood films, either directly or from the buffy coat in which the leukocytes are con-

centrated, after centrifugation of citrated blood. If not found, examine material obtained by splenic puncture or more safely by puncture of the liver or sternal bone marrow. (They are found occasionally in excised lymph glands or aspirated gland juice.) Blood globulin markedly increased. Make formol-gel and antimony tests. Marked leukopenia with relative monocytosis. Differentiate from brucellosis, typhoid and paratyphoid fever, Banti's disease, chronic malaria, leukemia.

Ketosis. Examine urine for ketone bodies. Test pH and titratable acidity of urine. Test for acidosis (q.v.).

Lead Poisoning. If exposure is recent, examine urine for lead. Blood shows early increase in reticulocytes and many stippled cells; later anemia, in acute cases severe and hemolytic in type. Fragility of red cells decreased. Often neutrophilic leukocytosis. Examine urine for albumin and casts. In late cases test renal function.

Leishmaniasis. See Kala-azar, Oriental sore, and Espundia.

Leprosy. Diagnosis depends upon demonstrating leprosy bacilli in smears stained by Ziehl-Neelsen method. Decolorize lightly with 20 per cent aqueous H_2SO_4. Morphology characteristic. Usually abundant in material from granulomas or scrapings from ulcers, especially from nose. If no lesions are evident, examine scrapings from nasal mucous membrane or skin clips from ear lobe. Examine blood during febrile periods; make thick films, dehemoglobinize and stain as above, or stain films from sediment after digestion with alkali. Cultures and animal inoculation useless except to exclude tuberculosis. Serologic reaction for syphilis in the blood positive in about 60 per cent of the cases.

Leukemia, Acute. Total leukocyte count ranges from normal to 150,000, occasionally much reduced. Diagnosis depends upon presence of many primitive leukocytes with nongranular basophilic cytoplasm and nuclei showing fine chromatin network and nucleoli. Progressive anemia with normoblasts. Reduced platelet count. Prolonged bleeding time. Increased basal metabolic rate. Marrow shows marked increase in primitive type of leukocyte.

Leukemia, Chronic Lymphatic. Leukocyte count usually from 20,000 to 100,000, with 75 to 99 per cent small lymphocytes, a few lymphoblasts and Rieder cells. May be a few myelocytes. Progressive anemia. Normoblasts rare. Basal metabolic rate increased. Blood uric acid high. Marrow (biopsy or sternal puncture) shows increase in lymphocytes.

Leukemia, Chronic Myelogenous. Total leukocyte count usually from 200,000 to 500,000, with a large proportion of myelocytes. In terminal stage many myeloblasts. Progressive anemia. Normoblasts nearly always present, often numerous. Platelets increased. Basal metabolic rate increased. Blood uric acid high. Examine sternal marrow.

Leukemia, Monocytic. Leukocyte count ranges from normal to 400,000 with 20 to 90 per cent monocytes and monoblasts. Examine fresh supravitally stained films as well as fixed films. Myelocytes usually present, may be numerous. Usually marked anemia and reduced platelets. Examine sternal marrow.

Liver, Necrosis of. Test liver function (see p. 873). Bilirubin in blood increased. Urobilinuria. In advanced stages shows: Increase in ratio of NH_3/urea in urine, with leucin and tyrosin crystals, usually only after concentration. High nonprotein nitrogen with low urea in blood. Hypoglycemia. Decreased fibrinogen. Prolonged prothrombin time. Decreased sedimentation rate. Acidosis may occur.

Lymphogranuloma Inguinale. Frei test.

Madura Foot. Discharge may contain fish-roe granules which show mycelium and peripheral clublike structures. Culture on Sabouraud's agar.

Malaria. Examine thick and thin films stained by Giemsa's or Wright's stain. To identify an object as a malarial parasite in stained films, one should be able to make out at least two of three characters: (1) chromatin; (2) bluish or greenish cytoplasm; and (3) pigment. Crescents are diagnostic for malignant tertian, equatorial banding for quartan. Marked irregularity of outline of parasite and the presence of Schüffner's (reddish) dots in cytoplasm of red cells indicates benign tertian. Leukopenia with mono-

cytosis. Leukocytosis during the paroxysms. Anemia of hemolytic type. Plasma globulin increased. Almost all show positive reaction to serologic test for syphilis during febrile periods. May try provocative procedures. In special cases make sternal (or splenic) puncture.

Malignant Neutropenia. Leukopenia, becoming extreme, with disappearance of granulocytes. Red cells, platelets, coagulation factors usually normal. Granulocytes in marrow often greatly reduced. Make blood culture to exclude sepsis. Stain films for Vincent's organisms. Differentiate from sepsis, acute leukopenic leukemia, and aplastic anemia. Examine sternal marrow.

Measles. Blood shows neutrophilic leukopenia. Diazo reaction in urine usually precedes eruption and is not found in German measles. (For use of convalescent serum, see p. 212.)

Melanosarcoma. Urine usually turns black after exposure to the air because of the formation of melanin. (This occurs, rarely, in other conditions, e.g., alkaptonuria.) Does not reduce Benedict's solution.

Meningitis, Lymphocytic Chorio. Spinal fluid shows increased pressure, slightly increased globulin, 50 to 2000 lymphocytes, meningitic colloidal gold curve, sterile cultures. Inoculate guinea pig subcutaneously, or mice intracerebrally. Virus-neutralizing antibodies in blood after sixth week. Differentiate from encephalitis, poliomyelitis, tuberculous meningitis, neurosyphilis.

Meningitis, Meningococcus. Blood cultures often positive in early cases and in simple meningococcemia. Spinal fluid purulent, under high pressure. Culture *immediately* on warm blood agar. Examine stained film for Gram-negative intracellular or extracellular diplococci. Smooth virulent strains show capsular swelling with specific antiserum. Neutrophilic leukocytosis. Differentiate from septic meningitis, tuberculous meningitis, poliomyelitis, encephalitis, benign lymphocytic choriomeningitis. Detect carriers by cultures from posterior nasopharynx.

Meningitis, Tuberculous. Spinal fluid clear or opalescent, shows increased pressure, increased globulin, lymphocytic pleocytosis, decreased sugar, markedly decreased chlorides. Stain for tubercle bacilli in fibrin web which forms on standing or centrifuged sediment after alum flocculation (p. 755). Make cultures or inoculate guinea pig. Differentiate from benign lymphocytic choriomeningitis, encephalitis, poliomyelitis.

Mercury Poisoning. Examine urine or gastric contents for mercury. Examine urine for volume (oliguria), albumin, casts, renal epithelium. Determine blood nonprotein nitrogen or urea, and creatinine. If severe, test for acidosis. Renal function tests show impairment.

Myeloma, Multiple. Bence-Jones protein usually found in urine. High plasma globulin (up to 9 per cent), even without Bence-Jones proteinuria. May show high blood calcium and low phosphorus. Anemia, occasionally erythroblastic, with leukocytosis and myelocytes. Roentgenogram.

Myiases. To identify fly larvae, make rearings to adults, or examine breathing slits on posterior stigmata of larvae.

Myxedema. Basal metabolic rate retarded (to —40 per cent). Blood shows high cholesterol, low glucose. Low flat glucose-tolerance curve. Anemia, either macrocytic or hypochromic microcytic in type. Often lymphocytosis.

Nephritis, Acute and Chronic. Examine urine, especially for albumin, casts, blood and pus cells. The presence of blood and red cell casts indicates an acute process. In acute cases look for streptococcal throat infections. (See p. 37.) Test renal function. Determine blood nonprotein nitrogen or urea, and if high, creatinine or phosphorus. If impaired, determine blood chloride or bases, and test for acidosis. Anemia common in advanced stages. (See "Nephrosis.")

Nephrolithiasis. Examine urine for blood and pus. The presence in *freshly* voided urine of clumped calcium oxalate or uric acid crystals is suggestive. Examine urine

for cystin crystals. Make culture to determine whether pyelitis is present. Roentgenogram. Determine Ca and P in blood to exclude hyperparathyroidism.

"Nephrosis." "Nephrotic" Stage of Glomerular Nephritis. Examine urine. Oliguria, high fixed specific gravity, marked albuminuria, many casts, epithelial cells with doubly refractile fat droplets, pus cells, but few or no red cells. Chloride excretion reduced. Phthalein excretion normal. Blood cholesterol very high, plasma albumin reduced, A/G ratio inverted. Chlorides may be high. No nitrogen retention. Reaction to Congo red test positive. Sedimentation rate much accelerated. Basal metabolic rate retarded. Anemia common. Look for evidence of intercurrent infections.

Ochronosis (Alkaptonuria). Urine contains homogentisic acid, which turns black on standing exposed to the air (especially if alkaline). Reduces Benedict's solution but not bismuth salts, and is not fermented by yeast. Rare anomaly of metabolism.

Onchocerciasis. Aspirate fluid from a nodule and look for motile microfilariae in fresh preparation, or stain by Giemsa. If negative, excise a nodule and look for adult worm or (very numerous) microfilariae. Clip off a bit of skin or conjunctiva, shake up in a few drops of salt solution and look for microfilariae. (Microfilariae are not usually present in the blood.) Reaction to complement-fixation test positive with special antigens.

Ophthalmia Neonatorum. See Gonococcus infection.

Oriental Sore. Examine scrapings from base of ulcer for leishmanial bodies. Stain with Wright's or Giemsa's stain. Preferably obtain material by aspiration by puncture near edge of ulcer. If bacterial contamination can be avoided, culture on special medium.

Oroya Fever. Acute, rapidly developing hemolytic anemia. Rodlike organism, *Bartonella bacilliformis,* in red cells.

Osteomalacia. Either blood calcium or phosphorus (or both) usually decreased. Blood phosphatase increased. Roentgenogram.

Otitis Media. Neutrophilic leukocytosis. Make smears from discharge and culture on blood agar. Make blood culture.

Pancreatic Disorders. Examine feces for undigested starch, muscle fibers, and abnormal amounts of total fat and neutral fat. Examine duodenal contents, especially for enzymes. (See p. 867.) Test urine for sugar. Determine blood sugar and diastase. Increased diastase in urine. Make glucose-tolerance test.

Paragonimiasis. Examine fresh sputum for light-yellow, operculated ova, averaging 90 by 65μ. Also for pus, blood, elastic fibers. Exclude tuberculosis. Ova in feces in about 40 per cent of cases.

Paratyphoid Fever. Examine as for typhoid fever.

Pertussis. In early stages, examine stained smears from sputum for small, oval, Gram-negative, bipolar-staining bacilli. Culture sputum (or use "cough plate" method) on Bordet-Gengou medium. Usually a very marked lymphocytosis.

Piedra. Examine hairs for small gritty masses which consist of spores arranged like mosaics about hairs.

Pituitary Disorders. In hyperpituitarism (acromegaly) there may be polyuria and glycosuria associated with hyperglycemia and reduced glucose tolerance. In hypopituitarism (dystrophia adiposogenitalis, Fröhlich's syndrome) blood sugar is low, glucose tolerance increased. Basal metabolic rate slightly retarded (but normal if calculated on basis of the ideal weight of the individual).

Plague, Bubonic Type. Examine material obtained by gland puncture for *P. pestis.* Stain smears, culture, and inoculate a mouse or guinea pig to identify. *Pneumonic type:* Examine the thin, watery, blood-tinged sputum in the same way. To obtain pure culture, inoculate on unbroken skin or nasal mucosa of animal. *Septicemic type:* Make blood culture. *P. pestis* may be sufficiently numerous to be found in blood films. Leukocytosis. Differentiate from tularemia.

Pleural Fluid. Determine specific gravity, albumin content, total cell count. Centrifuge a small portion collected in citrated salt solution, treat sediment with 1 per cent

formalin, centrifuge again, and stain sediment by Gram's and Ziehl-Neelsen stains for bacteria and by Wright's stain for differential count. Culture on blood agar and inoculate a guinea pig. In tuberculosis the fluid usually shows a lymphocytosis. (See p. 760.)

Pneumonia, Acute Lobar. Isolate pneumococcus from sputum (or material from lung puncture) by culture and mouse inoculation. Determine type directly from sputum by Neufeld's "Quellung" reaction; confirm with culture later. Make blood culture (often positive early; late in disease indicates a bad prognosis). Marked neutrophilic leukocytosis with shift to the left, and reduction in eosinophils. Low count indicates poor resistance. Urine chlorides much reduced. Blood chlorides reduced, nonprotein nitrogen high if oliguria is marked.

Pneumonia, Atypical. Make culture and stain film of sputum to exclude bacterial infection. Leukocyte count not increased. Examine serum at intervals for "cold" agglutinins.

Poliomyelitis. Examine spinal fluid. (See p. 205.) Culture on blood agar to exclude ordinary forms of meningitis. Moderate neutrophilic leukocytosis at onset.

Polycythemia. High red cell count (over six million) with increased blood volume. Increased viscosity. Evidences of active red cell regeneration, neutrophilic leukocytosis with often a few myelocytes, and increased platelets. High blood calcium. Basal metabolic rate often accelerated. In selected cases test renal function and liver function.

Pregnancy. Increased sedimentation rate. Reaction to Friedman or Aschheim-Zondek test positive. (See p. 881.)

Pregnancy, Toxemia of. Examine urine for evidences of nephritis. Test for ketone bodies, ratio of NH_3/urea and other evidences of acidosis. Tests for renal function and liver function.

Prostatic Hypertrophy. Examine urine for albumin, casts, and evidences of infection. Make culture. Test nonprotein nitrogen or urea and creatinine in blood. Usual tests of renal function not applicable except with catheterization.

Purpura Haemorrhagica (Thrombocytopenic). Platelet count reduced. Bleeding time prolonged. Clot retraction impaired. Reaction to tourniquet test positive. Coagulation time usually normal. May show secondary posthemorrhagic anemia. Leukocyte count variable.

Pyelitis. See Urinary tract infections.

Pyloric Obstruction. Examine fasting stomach contents for evidences of retention. Roentgenogram. Oliguria. Dehydration, with high red cell volume, high plasma proteins. Low blood chlorides and bases, and high nonprotein nitrogen and urea (normal creatinine), falling rapidly if diuresis is established. If HCl is vomited, alkalosis with high plasma bicarbonate and tetany. (See Gastric ulcer, Gastric cancer.)

Rabies. Keep dog, which has bitten patient, alive to observe symptoms. If dog has been killed, make smears from cornu Ammonis and stain by Giemsa's or Mann's stain for Negri bodies. (See p. 207.)

Relapsing Fever. Examine blood for spirochetes with darkfield or India-ink method, or in thick smears stained by Wright's or Giemsa's stain. (They may be absent from peripheral blood during afebrile period.) If not found, inoculate a mouse and examine its blood after 24 and 48 hours. Neutrophilic leukocytosis in acute cases. Differentiate from malaria, yellow fever, Weil's disease.

Rickets. Blood phosphorus usually low, calcium normal. In some cases calcium is low and phosphorus normal. Product of Ca $\times$ P is below 40 and often below 30. Blood phosphatase increased. Examine blood for anemia. Roentgenogram.

Rickettsial Infections. Inoculate nearly grown male guinea pig intraperitoneally with 1 ml. blood obtained during febrile period. Take temperature of pig regularly and watch for febrile reaction after 5 to 12 days. Watch for swelling of scrotum, and examine scrapings from tunica vaginalis for rickettsiae (Mooser bodies). Examine brain sections for small proliferative nodules and perivascular infiltrations. (See Table 2

p. 22.) Test serum of patient after seven days for agglutinins for Proteus OX_{19} (Weil-Felix). Moderate neutrophilic leukocytosis.

Rocky Mountain Spotted Fever. See Rickettsial infections.

Scabies. With the aid of a hand lens examine the infected skin for a black line which marks the tunnel for the parasite. The female can be found at the end of the tunnel and removed. Look for ova or mites in any stage of development in scrapings from skin.

Scarlet Fever. Neutrophilic leukocytosis of 12,000 to 15,000 with early increase in eosinophils. Make culture from throat on blood agar for hemolytic streptococci. Test for Schultz-Charlton reaction. Watch urine for evidences of nephritis. Make Dick tests and throat cultures on contacts.

Schistosomiasis. Examine urine (*S. haematobium*) and feces (*S. japonicum* and *S. mansoni*) for ova, particularly in masses of blood-tinged mucus. Blood in urine. Examine blood for anemia and eosinophilia. In late stages test liver function. Complement-fixation reaction positive with special antigen.

Scurvy. Reaction to tourniquet test of capillary resistance positive (as in purpura). Reduced excretion of cevitamic acid in the urine. May cause hypochromic anemia.

Septicemia. Make blood culture. Neutrophilic leukocytosis with shift to the left.

Smallpox. Initial leukopenia followed by neutrophilic leukocytosis in pustular stage. Monocytes increased. Try Paul's or McKinnon's inoculation tests. (See p. 195.)

Sporotrichosis. Culture on Sabouraud's agar or potato for eight days or more. Direct smears usually do not show organisms.

Sprue. Examine the frothy, pultaceous stools for undigested food and excess fat (25 to 40 per cent), chiefly fatty acids. Make gastric analysis (occasionally an achlorhydria). Examine blood for anemia, usually macrocytic, like pernicious anemia; occasionally hypochromic. Blood calcium reduced. Glucose tolerance curve has flat peak.

Syphilis. *Primary stage:* Look for *T. pallidum* in serous exudate from chancre in darkfield preparations (or stained films). If negative, and ulcer is healing, examine juice aspirated from regional bubo.

Secondary, tertiary and *latent* cases: Make complement-fixation or flocculation tests. Examine serous fluid expressed from secondary lesions by darkfield. Examine spinal fluid if clinical evidence of disease of the nervous system is present; and in all cases before treatment is stopped.

Tetanus. Inoculate white mouse or guinea pig and make anaerobic cultures from curettings from the wound. (See p. 70.) Rarely found in smears. Inject filtrate from culture into two guinea pigs, one of which should be protected by injection of antitetanic serum.

Thrombocytopenia. See Purpura haemorrhagica.

Thrush. Make scrapings from lesions and examine in 10 per cent KOH solution. The organism, *Candida albicans,* may be cultivated on Sabouraud's medium. It slowly liquefies gelatin and blood serum and acidifies and clots milk. In cultures there are budding yeastlike forms and mycelial threads.

Thyroid Disorders. See Exophthalmic goiter and Myxedema.

Transfusion. To select donor, secure individuals of the same blood group (or group O), and match the serum of the recipient with the cells of the donor and vice versa. Test for Rh factor in all women, and in men receiving repeated transfusions. Exclude syphilis by Kolmer or Kahn test and by physical examination. Exclude malaria by history and stained thick blood films.

Trichiniasis. Usually high leukocytosis and eosinophilia. Secure suspected meat, examine for encysted larvae in press preparations, or digest in artificial gastric juice, and collect larvae in Baermann apparatus (see p. 648). May feed meat to rat or mouse and examine muscles similarly after 10 days. During second or third week take 5 to 10 ml. blood in dilute acetic acid and examine sediment for larvae. After second week excise a

bit of muscle from deltoid or pectoralis near insertion and examine. Make intracutaneous test with Bachman antigen.

Trypanosomiasis. Examine blood or gland juice for trypanosomes in fresh preparations or stained thick films. When sparse, concentrate in blood by centrifugation and make films from leukocyte layer (see p. 522). If not found, inoculate a rat or guinea pig intraperitoneally with blood, gland juice, or emulsion of excised gland and examine blood at intervals. Reaction to formol-gel test often positive. In lethargic stage examine spinal fluid for parasites. Cell count and globulin increased. Differentiate from kala-azar, malaria, syphilis.

Tuberculosis. Make acid-fast stain of smears from sputum, fasting stomach contents, feces, or urinary sediment. If necessary, first concentrate by digesting in alkali or antiformin and centrifuging. Culture on special egg-yolk media, or blood agar to which a few drops of glycerin have been added. Inoculate guinea pig. Blood cultures may be positive in miliary tuberculosis. High monocyte-lymphocyte ratio indicates progressive lesion. Positive diazo reaction in urine an unfavorable sign. In special cases make intracutaneous tuberculin tests. Sedimentation rate increased in proportion to activity of disease.

Tularemia. In the early stages inoculate mouse or guinea pig with material from local lesion or regional glands, or with blood. At autopsy look for characteristic lesions (small caseous foci in organs) and make cultures from blood and organs on glucose cystin blood agar. After the first week test blood for agglutinins. Differentiate from Brucellosis, plague.

Typhoid Fever. Neutrophilic leukopenia; relative lymphocytosis; eosinophils reduced or absent. Blood culture usually positive during the first week, later less frequently obtained. Culture urine and feces on MacConkey, S. S., and bismuth sulfite media. Make agglutination test after 7 to 10 days. In suspected carriers culture urine and feces or duodenal contents. Differentiate from paratyphoid fever, brucellosis, malaria, typhus, miliary tuberculosis, liver abscess, kala-azar.

Typhus Fever. See Rickettsial infections.

Undulant Fever. See Brucellosis.

Urinary-tract Infections. Examine sediment immediately in hanging drop and in films stained by Gram's method. Collect specimen with sterile precautions or by catheter and culture on MacConkey and blood agar plates. In special cases search for tubercle bacilli by stain and culture and confirm by guinea-pig inoculation. Digest sediment in alkali if abundant or contaminated.

Yellow Fever. Early neutrophilic leukocytosis which in a few days falls to normal or below. Increasing albuminuria with granular and epithelial casts from the first or second day. Oliguria or anuria in fatal cases. Bile pigments are present in blood and urine in increasing amounts from the second or third day. Inject blood of patient (during the first three days intracerebrally into mice. Serum of cases after recovery shows lifelong protective power. Differentiate from severe malaria, blackwater fever, infectious jaundice, relapsing fever, dengue, influenza.

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